The ecological genetics of gynodioecy in *Silene acaulis* L. (Caryophyllaceae): spatial sex structure and inbreeding depression

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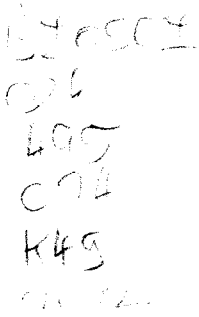

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ABSTRACT

Gynodioecy, the co-occurrence of females and hermaphrodites, is considered an intermediate step in the evolution of separate sexes in flowering plants. Highly variable female frequencies among populations suggest structuring of sex determining genes and differences in the relative fitness of females and hermaphrodites as seed parents. I investigated spatial variability in sex ratio and the effects of inbreeding on offspring quality in *Silene acaulis*. Female frequencies varied among populations from 0.32 to 0.69, and most were at temporal equilibrium. Females were significantly clumped within two of six populations. Females produced from 4 to 27 times as many fruits as hermaphrodites. Self-pollination of hermaphrodites reduced offspring survivorship and growth by an average of 67% compared to outcrosses. Overall, spatial variation in female frequency suggests a role of founder events and local seed dispersal. Low fruit production and reduced quality of inbred offspring suggest hermaphrodites may be functioning primarily as pollen donors.

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GENERAL INTRODUCTION

The evolution of separate sexes from an ancestral state of hermaphroditism is a central issue in evolutionary biology. The great diversity of breeding systems and sex expression in higher plants make them ideally suited to addressing this issue. Among flowering plants, gynodioecy is a form of gender dimorphism characterized by the coexistence of female and hermaphrodite individuals within populations (Darwin 1877). Approximately 7% of all known angiosperm species are gynodioecious (Richards 1997), and this breeding system is thought to be an important intermediate step in the evolution of separate male and female plants (Lloyd 1974, Charlesworth and Charlesworth 1978, Desfeux *et al.* 1996).

Variation in the population sex ratio is widespread among many gynodioecious species. Because sex expression is heritable, variation in the frequency of females may primarily reflect differences in the distribution and diversity of sex determining genes (Gouyon and Couvet 1987, Manicacci *et al.* 1997, Olson and McCauley 2001). In most species, females occur when maternally inherited cytoplasmic male sterility (CMS) genes located on the mitochondrial genome inhibit pollen production (Charlesworth and Laporte 1998, Taylor *et al.* 2001). Autosomal restorer genes can cancel the effects of CMS and restore male function, leading to hermaphrodite individuals. Because CMS genes are maternally inherited and dispersed only through seeds, they are expected to show spatial patchiness both within and between populations. This is in contrast to autosomal genes such as restorers which disperse more widely through both seeds and pollen. Therefore, restricted dispersal of CMS by seeds may lead to spatial variation in

sex ratio, an outcome with potentially strong impacts on the fitness of individuals (McCauley and Taylor 1997, Graff 1999).

By using sex expression as a phenotypic marker, observations of the spatial and temporal distribution of the sexes can provide insight into the current and historical dynamics of dispersing CMS and restorers (Frank 1989). In addition, quantifying fitness traits allows for interpreting the consequences of sex expression for the reproductive output of individuals and populations. I use this approach in Chapter 1 to address sex ratio variation and its effects on fecundity and seed viability within and among six Alaskan populations of the gynodioecious plant, *Silene acaulis*. Specifically, I address the following questions: (1) Do populations differ in their sex ratio, and, if so, at what geographic scale? (2) Is sex expression spatially homogenous within populations? (3) Is there evidence for temporal instability in the sex ratio? (4) Do the sexes differ in fruit production? (5) Does female frequency correlate with the mean viability of offspring among populations?

Because females incur the loss of male function by not making pollen, they suffer a reduction in potential gene transmission compared to hermaphrodites. This is because females contribute their genes only through ovules, whereas hermaphrodites can contribute genes through both ovules and pollen. Consequently, for females to persist in a population, they must possess an advantage over hermaphrodites as seed parents (Lewis 1941, Lloyd 1974, Charlesworth and Ganders 1979). Females may achieve a fitness advantage by producing more seeds, seeds of higher quality, or both. The expression of deleterious genes following inbreeding may affect offspring quality and is thought to be

an important factor shaping the evolution of breeding systems (Lande and Schemske 1985, Charlesworth and Charlesworth 1978). Because the vast majority of gynodioecious species are self-compatible, self-fertilization by hermaphrodites may result in inbreeding depression in their seed offspring, whereas females are incapable of self-fertilizing. Additionally, both sexes are capable of biparental inbreeding with related individuals. Quantifying inbreeding depression and its effects on the relative fitness of the sexes is therefore a major goal for studies investigating gender evolution.

There may also be maternal effects on offspring quality that differ between the sexes, independent of inbreeding. Females and hermaphrodites may differ in their female function through patterns of resource allocation and seed provisioning (Eckhart and Chapin 1997, Poot 1997), sexual selection (Wilson and Burley 1983, Shykoff 1992), or sex-linked genes with fitness effects (e.g., de Hann 1997). Studies that compare inbred to outcrossed offspring of both females and hermaphrodites, thereby removing the confounding of inbreeding and maternal sex, are critical for understanding how these two factors act independently and interactively in determining offspring performance (Ashman 1992, Thompson and Tarayre 2000).

In Chapter 2, I report the results of a greenhouse experiment designed to assess the independent and interactive effects of inbreeding and maternal sex on offspring quality among four Alaskan source populations of *Silene acaulis*. I address the following questions: (1) Do offspring produced by self-fertilized hermaphrodites differ in quality compared to outcrossed offspring? (2) Do biparentally inbred offspring differ in quality compared to outcrossed offspring? (3) Do offspring from females and hermaphrodites

differ in their response to biparental inbreeding? (4) How do the effects of inbreeding and maternal sex differ among populations?

Silene acaulis (Caryophyllaceae), the moss campion, is a gynodioecious plant with a Holarctic distribution. Populations often show considerable spatial variation in the frequency of females (Hermanutz and Innes 1994, Delph and Carroll 2001). Females generally produce more offspring compared to hermaphrodites (Morris and Doak 1998, Delph and Carroll 2001), as well as offspring with a higher probability of survivorship (Shykoff 1988). The exact nature of the genetics of sex expression in *S. acaulis* is unknown, but several lines of evidence suggest cytoplasmic genes are involved. For example, female frequencies in populations are sometimes greater than 50% (the upper limit predicted by theory under purely nuclear inheritance of sex), offspring sex ratios may be highly biased towards the sex of the mother, and there is a history of cytoplasmic male sterility in the genus *Silene* (Desfeux *et al.* 1996, Charlesworth and Laporte 1998, Taylor *et al.* 2001).

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CHAPTER 1:

Spatial and Temporal Patterns of Male Sterility in the Long-Lived Gynodioecious Plant, *Silene acaulis* (Caryophyllaceae)[1]

Introduction

Spatial structure in plant populations has been an important focus of many ecological and evolutionary studies because of its potential to influence patterns of mating between individuals and hence the genotype of the resulting offspring (Sakai and Oden, 1983, Epperson and Clegg 1986, Hossaert-McKey *et al.* 1996, Kalisz *et al.* 2001). In gender dimorphic plant species with genetic sex determination, spatial variation in sex expression is a form of population structure that may result from sex-based differences in microsite preference (Meagher 1980, Dawson and Bliss 1989), sex-biased herbivory or mortality (Bawa and Opler 1978, Bierzychudek and Eckhart 1988, Allen and Antos 1993), or the distribution and dynamics of sex determining genes (Manicacci *et al.* 1996, Laporte *et al.* 2001, Olson and McCauley 2002). Spatial structuring of sex expression may occur at several scales, with differences observed among populations (Delph 1990, Taylor 1999, Olson 2001) and at a fine scale within populations (Sakai and Oden 1983, Manicacci *et al.* 1996, Laporte *et al.* 2001). Such structure may influence the evolution of gene frequencies, the reproductive performance of individuals (Sakai and Oden, 1983, McCauley and Taylor 1997, McCauley and Brock 1998, Graff 1999), and the population as a whole (Gouyon and Couvet 1987).

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Gynodioecy is a form of gender dimorphism exhibited by approximately 7% of all flowering plant species (Richards 1997) and is thought to be an intermediate step in the evolution of dioecy (Lloyd 1974, 1975; Charlesworth and Charlesworth 1978, Desfeux *et al.* 1996, Schultz and Ganders 1996). In gynodioecious species, both male sterile (hereafter referred to as female) and hermaphrodite individuals co-occur (Darwin 1877). Females generally produce more seeds and/or better quality seeds, presumably due to reallocation of resources saved by not producing pollen (Poot 1997, Ashman 1999). This fitness advantage over hermaphrodites is necessary for females to persist in a population because females suffer a reduction in potential gene transmission compared to hermaphrodites by not producing male gametes (Lewis 1941).

Variation in population sex ratio is widespread among gynodioecious plants. Studies have documented highly variable sex ratios among populations of *Beta vulgaris* (Laporte *et al.* 2001), *Bidens* spp. (Sun and Ganders 1986), *Hebe strictissima* (Delph 1990), *Silene acaulis* (Philipp 1997, Maurice *et al.* 1998), *Silene vulgaris* (Olson and McCauley 2002), and *Thymus vulgaris* (Belhassen *et al.* 1989, Tarayre and Thompson 1997). Sex ratio variation may reflect factors affecting female advantage such as differences in site quality (Krohne *et al.* 1980, Delph 1990, Ashman 1999), pollen limitation (Maurice and Fleming 1995, McCauley and Taylor 1997, McCauley and Brock 1998), or levels of self-fertilization and inbreeding depression in hermaphrodites (Lloyd 1975, Charlesworth and Ganders 1979, Sun and Ganders 1986, Schultz and Ganders 1996). In addition, because sex expression is heritable, variation in female frequency may primarily reflect differences in the distribution and diversity of sex determining

genes (Gouyon and Couvet 1987, Frank 1989, Manicacci *et al.* 1996, Laporte *et al.* 2001, Olson and McCauley 2002). In most gynodioecious species, females occur when maternally inherited cytoplasmic male sterility (CMS) genes inhibit pollen production (Charlesworth and Laporte 1998, Taylor *et al.* 2001). Autosomal restorer genes can cancel CMS and restore male function, leading to hermaphrodite individuals. Multiple CMS types have been shown to exist within many populations, each apparently inhibiting pollen production by a different developmental pathway, and each theoretically susceptible to restoration by one or more nuclear restorer loci (de Hann *et al.* 1997a, Charlesworth and Laporte 1998). Therefore, spatial structuring of sex determining genes by founder events and restricted dispersal may lead to variation among populations in sex ratio with a principally genetic basis (Gouyon and Couvet 1987, Manicacci *et al.* 1996, Laporte *et al.* 2001, Olson and McCauley 2002).

Limited pollen and seed dispersal may also spatially structure sex at a fine scale within a population. When females are the result of maternally inherited CMS genes, the sex ratios of seed families should be biased towards the sex of the mother (Gouyon and Couvet 1987). Indeed, this prediction is commonly observed in field pollinated families and experimental crosses (e.g., Taylor *et al.* 2001). When maternal plants disperse their seeds locally, bias in offspring sex ratios may result in non-random spatial associations of sex and the formation of sex-biased neighborhoods (Manicacci *et al.* 1996). Spatial structure of sex at the neighborhood level may significantly affect the seed fitness of gynodioecious plants through pollen limitation, and may have important repercussions

for the relative fitness of females and hermaphrodites (McCauley and Taylor 1997, McCauley and Brock 1998, Graff 1999).

The spatial structure of sex expression may also have an important temporal component, especially if the sex ratio is not at equilibrium. For instance, CMS genes may invade a population before their restorer alleles, causing significant female bias in the sex ratio (Frank 1989). Over time, as restorers arrive by either mutation or migration, the sex ratio is expected to become more hermaphrodite biased until another CMS invades, resulting in repeated sharp increases in the frequency of females (Frank 1989). Another mechanism hypothesized to cause nonequilibrium in sex ratio occurs when inverse frequency dependent selection maintains a stable polymorphism at both CMS and restorer loci, causing the frequency of restoration (and hence the prevalence of females) to repeatedly cycle over time (Gouyon *et al.* 1991). Spatial variation in the sex ratio, represented by differences among populations, would then result from asynchrony in the temporal cycling of sex determining genes (Gouyon *et al.* 1991). Few studies have assessed the degree of equilibrium or nonequilibrium in the frequency of females, probably due to the difficulty in obtaining multigenerational data from herbaceous plants (but see Belhassen *et al.* 1989, de Hann *et al.* 1997b).

Variation in the sex ratio may have important consequences for differences in mean fitness among populations. Because females are obligate outcrossers, their offspring should be heterozygous at a higher proportion of loci compared to offspring of hermaphrodites, which may be the products of self-fertilization. Therefore, theory predicts the proportion of heterozygotes in the entire population will be greater when

female frequency is high (Gouyon and Couvet 1987). To the extent that heterozygosity and fitness are positively related (Hartl and Clark 1989, but see Britten 1996), female biased populations on average should produce offspring of greater viability compared to offspring from hermaphrodite biased populations.

By using sex expression as a phenotypic marker, observations of the spatial and temporal distribution of the sexes can provide insight into the current and historical balance between CMS genes and restorers (Frank 1989). Quantifying fitness traits then allows for interpretation of the consequence of these genes for the reproductive output of individuals and populations. I use this approach in the current study to address the following questions regarding the spatial and temporal structure of sex expression within and among Alaskan populations of the long-lived, gynodioecious *Silene acaulis* (L.) Jacq. 1) do populations differ significantly in their sex ratio and, if so, at what geographic scale, 2) is sex expression spatially homogeneous within populations, 3) does the sex ratio within populations differ among different sized individuals (as a proxy for age), reflecting temporal changes in sex ratio, 4) do females achieve a fitness advantage over hermaphrodites, and 5) does female frequency correlate with the mean viability of offspring among populations?

Methods

Silene acaulis (L.) Jacq. (Caryophyllaceae) is a long-lived, gynodioecious perennial distributed widely throughout arctic-alpine North America and Europe. Populations often occur on disturbed sites such as glacial moraine and alpine talus

(Benedict 1989, Morris and Doak 1998). Individuals form compact cushions of rosettes and grow radially over time. Cushions have single taproots and produce many fragrant pink flowers. The species does not root adventitiously or reproduce clonally (Shykoff 1988, Morris and Doak 1998, Delph and Carroll 2001). Hermaphrodites are self-compatible and highly protandrous, with each flower producing two whorls of five anthers that dehisce in sequence before three styles elongate. Females produce three elongated styles and have rudimentary anthers that lack pollen. A small number of gynomonoecious individuals that produce both hermaphrodite and female flowers are also observed, although these generally occur in low proportions (Shykoff 1988, Morris and Doak 1988, S. Keller personal observation). Most flowers are insect pollinated, and the principal pollinators are *Bombus* spp. with occasional visits by lepidopterans and dipterans (Delph and Carroll 2001, S. Keller personal observation). The exact nature of the genetics of sex expression in *S. acaulis* is unknown, but several lines of evidence suggest cytoplasmic genes are involved. Namely, female frequencies in populations are sometimes greater than 50% (the upper limit predicted by theory under purely nuclear inheritance of sex), offspring sex ratios display complex segregation of sex (Delph unpublished data, cited in Delph and Carroll 2001, S. Keller unpublished data), and there is a history of cytoplasmic male sterility in the genus *Silene* (Desfeux *et al.* 1996, Charlesworth and Laporte 1998, Taylor *et al.* 2001).

Spatial Variation in Sex Ratio

During the summer of 2001, I established a single permanent plot in each of six study populations located in each of three distinct mountain ranges in Alaska, USA (Table 1.1; Figure 1.1). Plots were rectangular and ranged in size from 100 m^2 to 384 m^2 in order to include a large sample of individuals in populations that varied greatly in plant density (Table 1.1). I mapped all individuals within the study plots and measured the size of each cushion (± 1 cm), defined as the diameter of the longest horizontal axis (Morris and Doak 1998). Measurements were confined to cushions ≥ 5 cm in diameter because the absence of flowering in smaller cushions prohibited sex determination. Plants were numbered and marked with toothpick flags inserted into the cushion. I followed plants throughout the growing season and recorded sex as they flowered.

I recorded fruit production of mapped plants for two of these study populations (BG1 and ES2) at the end of the 2001 growing season. Monitoring of fruit set among all six geographically distant populations was not possible due to time and resource constraints. These two populations provide a useful comparison because each showed female bias in the sex ratio, but one exhibited spatial structuring of individuals while the other did not (Figure 1.2).

Sex Ratio Variation Across Size Classes

Previous studies have shown a positive relationship between cushion diameter and estimated age in *S. acaulis* (Benedict 1989, Morris and Doak 1998). Using data on annual diameter increases, estimated linear growth rates vary from 0-2 cm · yr^{-1} in

Colorado populations (Benedict 1989), to 0.086-0.163 cm · yr^{-1} in Alaska (Morris and Doak 1998), to 0.71-0.74 cm · yr^{-1} in the Alps and Greenland (R.E. Beschel cited in Benedict 1989), showing variation with latitude and regional conditions. Growth rate curves vary with cushion size, with a general pattern of slow growth in small cushions, more rapid growth in medium sized cushions, and a decline in growth rate among the largest plants (Benedict 1989, Morris and Doak 1998).

Given the positive relationship between cushion diameter and age in *S. acaulis*, I investigated temporal changes in sex ratio within each population by measuring the frequency of each sex across five size classes. Size classes were delimited as follows: class 1: 5-7 cm, class 2: 8-10 cm, class 3: 11-13 cm, class 4: 14-19 cm, class 5: 20 cm and larger (max = 58 cm). These roughly correspond to size classes 6 through 12 of Morris and Doak (1998). Reducing bin size and thereby increasing the number of size classes did not qualitatively affect the results. The mean estimated age of a plant residing in Morris and Doak's (1998) largest size class ($\geq$ 20 cm) is 300 years, with plants in excess of 500 years of age possible. Morris and Doak (1998) estimate the age at first reproduction at 24.7 (16.4, SD) years of age for females and 34.4 (21.4) for hermaphrodites, corresponding approximately to my size class 1. Thus, the size distribution of females and hermaphrodites within populations of *S. acaulis* should reflect sex ratio evolution over many overlapping generations.

Offspring Viability

To assess the relationship between population sex ratio and mean progeny viability, I collected five fruits from 11 to 18 haphazardly chosen maternal plants within each population at the end of the 2000 growing season. A total of 94 maternal seed families across the six study populations were collected. Because flowering had largely ceased by this time, I could determine sex for only 15 of these maternal plants (11 females and four hermaphrodites). While I did not consciously sample plants with regard to fruit production, females are much more likely to produce fruit compared to hermaphrodites (see Results section), and therefore this sampling protocol may have been biased towards including a disproportionate number of female plants, especially in female biased populations. For this reason, the value of the data is in comparing seed viability among populations with different sex ratios and not among individuals of different sex within populations. Fruits were placed in cold storage until January 2000, when seeds were counted and pooled within each maternal parent. Seeds were placed in Petri dishes on wetted filter paper and allowed to germinate at room temperature under constant light. Germination was scored when the radicle penetrated the seed coat, at which time the seedling was transplanted into a 284 ml cup filled with a potting medium (1:1:1 Coco-Grow:perlite: vermiculite) and placed on a greenhouse bench under a 24 hr photoperiod of natural and artificial light. Plants were watered and fertilized on a regular basis. I quantified survival through the establishment period by recording seedling mortality 4 weeks after planting.

Statistical Analyses

I used log-likelihood (G) to test differences in sex ratio among populations and to test associations between sex and size class within each population (Zar 1999). To determine if sex ratio showed directional change across size classes, I used Cochran-Armitage tests of trend in frequency data (PROC FREQ TREND OPTION: SAS Institute, 1988).

To assess the degree of non-random sex association within populations, I performed spatial autocorrelation on join counts (Sokal and Oden, 1978, Sakai and Oden 1983, Epperson and Clegg 1986, Real 1996). Join counts are a useful measure of autocorrelation for discrete data such as sex and are defined as a connection between two individuals, each possessing a value of the discrete variable within a given distance class (Sokal and Oden 1968, Real 1996). It is important to note that the analysis of spatial autocorrelation does not address the question of how individuals are spatially dispersed, but rather how a character state (in this case, sex expression) is spatially arranged among those individuals. Given the two sexes I scored in each population, three classes of joins were possible: female-female, female-hermaphrodite, and hermaphrodite-hermaphrodite. I excluded non-flowering individuals from the analyses. Join-counts for sex were tabulated at 0.25 m distance intervals and tested against the null hypothesis of no association between join type and distance class. Expected values and variances for the number of each join type in a given distance class were generated by 1000 permutations of the dataset (Matlab Student Version 5.3, The Mathworks, Inc., 1999). For each permutation, sex was sampled without replacement and randomly reassigned to a mapped

location of an individual. Therefore, the procedure tests the spatial association of sex given the observed spatial pattern of individuals while keeping the overall sex ratio in the population constant. A standard normal deviate (*Z*-score) was constructed by subtracting the expected number of joins generated by the 1000 permutations from the observed number of joins and dividing this difference by the standard deviation of the expected number of joins. Because the tests of association of sex within each population are based on the same individuals at multiple distance classes, they are not independent. Therefore, overall significance ($\alpha = 0.05$) of the autocorrelation in each population was assessed using the Bonferroni criterion (Sakai and Oden 1986, Rice 1989).

I modeled the probability that a flowering plant in BG1 and ES2 set at least one fruit using a log-linear analysis (PROC LOGISTIC: SAS Institute, 1988), with population and sex as grouping variables. For these same two populations, I used fixed effects analysis of covariance (ANCOVA) to test differences in fruit production between the sexes and populations while controlling for cushion diameter (PROC GLM: SAS Institute, 1988). Data were log-transformed prior to ANCOVA to better meet assumptions of normality and homoscedasticity.

The relationship between mean progeny viability and population sex ratio was quantified using Pearson's correlation (PROC CORR: SAS Institute 1988). Progeny viability was evaluated separately for the proportion of seeds germinating and for the proportion of seedling mortality. Since the hypothesis explicitly predicts viability should increase with the proportion of females in the population, I used one-tailed tests to assess

significance. Data were population means using each maternal family as the unit of observation.

Results

Spatial Variation in Sex Ratio

Female frequency varied among the six study populations from 0.32 to 0.69 (G = 69.34, df = 5, $P < 0.0001$; Table 1.1), and showed no apparent geographic pattern (Figure 1.1). For example, the frequency of females in populations BG1 and BG2, which are separated by only 0.4 km, was 0.69 and 0.52 respectively ($G = 6.45$, df = 1, $P = 0.01$). Similarly, ES1 and ES2 have a respective frequency of females of 0.39 and 0.55 (G = 7.41, df = 1, $P < 0.01$) and are separated by a distance of 1.0 km. Likewise, populations separated by large distances also showed significant variation in sex ratio, with no consistent relationship between geographic distance and female frequency ($r = -0.19$, P = 0.49; Figure 1.1).

Within populations, I also observed nonrandom spatial association of sex. Two populations, ES2 and TMS, showed significant spatial autocorrelation of sex following Bonferroni correction, while the other four populations showed no significant autocorrelation (Figure 1.3). Both ES2 and TMS showed positive autocorrelation of female-female joins and negative autocorrelation of female-hermaphrodite joins up to a distance of about 1.25 m. Positive spatial association of females occurred without regard to their overall frequency within these two populations (Table 1.1). All other populations showed either weak trends of autocorrelation (BG2) at small distances or spatial

randomness (BG1, ES1 & KCT). Thus for ES2 and TMS, given the observed population-wide sex ratio, female individuals tend to be nonrandomly clumped into patches a bit larger than a meter while hermaphrodites are spatially distributed at random.

Sex Ratio Variation Across Size Classes

In four of the six populations, I detected no association between sex ratio and size class ($P > 0.15$; Figure 1.4). In contrast, the frequency of the sexes varied across the size classes within the other two populations, ES1 and BG1 (ES1: $G = 9.99$, df = 4, $P = 0.04$; BG1: $G = 10.05$, df = 4, $P = 0.04$). For both populations, the proportion of females was less in smaller size classes (Cochran-Armitage trend tests, ES1: $Z = 2.61$, $P = 0.009$; BG1: $Z = 1.90$, $P = 0.06$), suggesting that female frequency has decreased over time in these two populations. In all populations, the number of individuals (including vegetatives) decreased with increasing cushion size (Figure 1.5). The frequency of individuals within different size classes did not differ significantly among populations ($G = 22.52$, df = 20, $P = 0.31$), suggesting similar patterns of establishment and survivorship exist among the populations.

Fruit Production and Offspring Viability

In the two populations for which fruit production was measured, BG1 and ES2, the odds that a flowering female set at least one fruit was 18.24 times greater than that of a hermaphrodite fruiting (Logistic regression: Wald $\chi^2 = 95.29$, df = 1, $P < 0.0001$). Fruit production also differed between the populations (Wald $\chi^2 = 21.32$, df = 1, $P < 0.0001$); a

flowering plant in BG1 was over 4 times more likely to set fruit than a flowering plant in ES2 (adjusted odds ratio, 95% C.I. = 4.03, 2.27 – 7.17). There was no significant interaction between population and sex in the likelihood of setting fruit ($P > 0.25$)

The number of fruits produced per plant increased significantly with cushion size (Figure 1.6). A significant three-way interaction indicated this trend was stronger for females than hermaphrodites, though the relationship varied between the two populations (Table 1.2). Hermaphrodites in ES2 produced very few fruits even among the large diameter individuals, while some of the larger hermaphrodites in BG1 produced some fruits (Figure 1.6). Averaged across all cushion diameters, female advantage in the quantity of fruits produced (mean female fruit production / mean hermaphrodite fruit production) was 4.03 in BG1 and 27.46 in ES2.

Across populations, the frequency of females covaried with the viability of open-pollinated seed. The population mean (± SE) proportion seed germination varied from 0.08 ± 0.03 to 0.64 ± 0.07, and increased with female frequency ($r = 0.86$, $P_{1\text{-tail}} = 0.014$; Figure 1.7). Mean seedling mortality was also variable across populations (mean ± SE: 0.06 ± 0.02 to 0.33 ± 0.06) and showed a non-significant tendency to decrease with an increasing frequency of females ($r = -0.59$, $P_{1\text{-tail}} = 0.108$; Figure 1.7). When combined into a multiplicative estimate (proportion germination * [1-proportion mortality]), offspring viability through the seedling establishment stage increased significantly with population female frequency ($r = 0.85$, $P_{1\text{-tail}} = 0.017$). Differences in seed germination between the sexes showed no differences (ANOVA: $F_{1,14} = 0.34$, $P = 0.57$) among those

plants where sex was known, indicating the relationship between viability and population sex ratio is not simply a sampling phenomenon.

Discussion

Spatial Variation in Sex Ratio

Alaskan populations of *S. acaulis* show considerable variation in sex ratio, ranging from 32% to 69% females, with large differences among populations separated by < 1 km (Figure 1.1). This suggests either the influence of heterogeneity in ecological factors differentially affecting survival of the sexes or genetic differentiation for sex determining genes among my study populations. Because sex ratios differed significantly, even among closely spaced populations that showed no obvious differences in habitat type, I am reluctant to emphasize a strong environmental role in generating these differences. Rather, three pieces of evidence lead me to conclude that sex ratio variability probably represents population genetic structure of sex determining genes: 1) occasional female bias in the population sex ratio, consistent with a cytoplasmic role in sex determination (Hermanutz and Innes 1994, Maurice *et al.* 1998, this study), 2) maternal inheritance of cytoplasmic genes combined with the likelihood of restricted dispersal through seeds, and 3) previous work showing a high degree of cytoplasmic genetic structure among local populations of other gynodioecious species (Laporte, *et al.* 2001, Olson and McCauley 2002). Other studies of *S. acaulis* have also shown variation in sex ratio among local populations. For example, among ten populations located along an 8 km ridge in Colorado, Delph and Carroll (2001) found the frequency of females was

significantly heterogeneous, varying from 23% to 39%. Maurice *et al.* (1998) studied 14 local populations in the French Alps and found sex ratios varied from 0% to 71% females. Among arctic populations, Philipp (1997) found the frequency of females in ten Greenland populations that were located within several kilometers of each other to vary from 41% to 91%. However, not all studies show sex ratio differences between closely spaced populations. For example, among five sites separated by between 0.3 to 4.0 km on Baffin Island, Canada, Hermanutz and Innes (1994) found female biased sex ratios of 72% to 80% that did not vary significantly from each other. In general, the above evidence does not refute a role of environmental conditions in favoring one sex over the other, but suggests that either ecological gradients and plant response must be very strong or that the observed variability in sex ratio has a principally genetic basis, in agreement with arguments formulated by Gouyon and Couvet (1987) and Gouyon *et al.* (1991).

Non-random sex structure of *S. acaulis* populations may also occur at a continental scale. Specifically, North American populations in Colorado show no instances of female bias. For example, among the studies of *S. acaulis* that report sex ratios from Colorado (Shykoff 1988, Delph and Carroll 2001), all 11 populations had frequencies of females less than 40%, compared to female biased sex ratios commonly reported at higher latitude sites in Canada, Greenland, and Alaska (Hermanutz and Innes 1994, Philipp 1997, Alatalo and Molau 1995, Morris and Doak 1998, this study). A possible explanation is that Colorado populations represent a disjunct group following the last glacial ice age and contain only a subset of the CMS diversity found within the main distribution of *S. acaulis* at higher latitudes. If appropriate restorers are present, limited

CMS diversity could prevent locally high frequencies of females from occurring due to lack of novel CMS types available to migrate into populations (Frank 1989). Testing this hypothesis requires additional sex ratio data from populations in the Rocky Mountains, along with an investigation of cytoplasmic diversity throughout the distribution of *S. acaulis*.

Within populations, clustering of females suggests a fine-scale spatial genetic structure similar to that observed in other gynodioecious species. Females were spatially associated in some (ES2 and TMS), but not all, populations (Figure 1.3). I attribute this pattern to restricted dispersal of seeds and/or pollen and therefore spatial variation in sex determining genes. Other studies have shown positive spatial autocorrelation of cytoplasmic genes within populations of gynodioecious species. For example, in a Virginia population of *Silene vulgaris*, Olson and McCauley (2002) demonstrated significant clumping of mtDNA haplotypes at the scale of meters. Further, Taylor *et al.* (2001) showed mtDNA haplotypes from this region possess a strong association with sex, suggesting they may represent distinct CMS types. Other studies have reported similar fine-scale cytoplasmic genetic structure in *Thymus vulgaris* and *Beta vulgaris* (Tarayre *et al.* 1997, Laporte *et al.* 2001).

While I do not know the spatial distribution of cytoplasmic genes within my populations, a plausible explanation for female patchiness in *S. acaulis* is the presence of a male sterile cytoplasm that lacks the appropriate restorer, causing sex expression to be temporarily under cytoplasmic control. Within populations of gynodioecious *T. vulgaris*, Manicacci *et al.* (1996) observed patches of females occurring particularly after

disturbance such as fire and found they often represent a single cytotype. They concluded that patches of females may represent founder events and temporary cytoplasmic control of sex expression. An alternative explanation for clustering of females in my study is that both a CMS gene and its complimentary nuclear restorers are present but highly spatially structured, with limited gene flow causing spatial "gaps" in restoration. To test this idea, one would have to perform controlled crosses using dams derived from areas of female bias and mate them with a variety of sires from throughout the population. Observing the segregating sex ratio of the offspring would address the question of whether the population contains restorers for that CMS type (Manicacci *et al.* 1997).

Assuming cytonuclear sex determination in *S. acaulis*, the lack of spatial association for sex expression in BG1, BG2, ES1 & KCT (Figure 1.3) suggests that either 1) pollen and seed dispersal has homogenized the distribution of CMS types and restorers, resulting in spatial randomness of sex expression, or 2) that CMS types are spatially structured, but dispersal of restorers through pollen flow is widespread enough to prevent clumps of unrestored offspring and thus female patchiness from developing. Contrasting the spatial structure of cytoplasmic and nuclear genetic markers could differentiate between these alternatives and has been a useful approach in studying gene flow in other gender dimorphic species (e.g., McCauley *et al.* 1996).

Sex Ratio Variation Across Size Classes

Within four of the six populations (BG2, ES2, KCT & TMS), the relative frequency of females and hermaphrodites was constant across the five size classes (Figure 1.4). If size is a good predictor of age in *S. acaulis*, as other studies have shown (Benedict 1989, Morris and Doak 1998), then this is evidence for temporal equilibrium in the sex ratio over several overlapping generations for the majority of my study populations. Several non-mutually exclusive explanations must be considered when interpreting this result. 1) Temporal patterns in sex ratio evolution across size classes may be obscured by stochastic events such as between-year variability in seed production and establishment, sex differential growth or shrinkage of cushions, or random events that caused greater mortality in one sex compared to the other. These could all produce demographic noise and obscure directional evolutionary change in the sex ratio. 2) These populations may be at equilibrium with purely nuclear control of sex expression. This can happen when a single CMS type occurs in a population and becomes fixed. Polymorphism at the restorer locus then causes sex expression to vary depending on the nuclear genotype of the individual (Gouyon *et al.* 1991, Manicacci *et al.* 1996). In this scenario, theoretical models predict the frequency of females to reach an equilibrium value in the presence of an appropriate nuclear restorer (e.g., Lewis 1941, Lloyd 1975). 3) The long generation time in *S. acaulis* undoubtedly results in many overlapping generations in each population, making evolutionary change in the frequency of sex determining genes very slow. This raises the question of how quickly the sex ratio is predicted to change over time if nonequilibrium processes are at work. Frank (1989) and

Gouyon *et al.* (1991) suggest that when female frequency rises above a threshold level, changes over a brief number of generations are to be expected. Based on Morris and Doak's (1998) estimates for the age at reproductive maturity in females (24.7 yr) and hermaphrodites (34.4 yr), and a conservative estimate of the oldest individuals in Alaskan populations being 300 yr, then the current data may represent approximately 8 to 12 overlapping generations, which would provide a reasonable time in which changes in sex ratio could occur.

For BG1 and ES1, there were proportionally fewer females in the smaller size classes, indicating that the sex ratio has become less female biased over time. This pattern would arise if female bias in the sex ratio caused by CMS genes was gradually eroded by the spread of nuclear restorers (Frank 1989). This is further supported by Belhassen *et al.* (1989), who found evidence for nonequilibrium processes generating variation among populations of *T. vulgaris*, where older populations had a lower female frequency than younger populations. The implication that initially high frequencies of females in young populations occurs due to founder events also receives support from Manicacci *et al.* (1996), who compared a population of *T. vulgaris* before and after disturbance. Prior to disturbance, the population contained 73% females and seven cytoplasmic haplotypes. Following a fire that killed all the individuals, the population was 100% females which all carried the same haplotype (Manicacci *et al.* 1996). These findings strongly suggest that female biased sex ratios may be a commonly observed but transitory state in gynodioecious populations subject to periodic invasion by new CMS types.

Fruit Production and Offspring Viability

Females outperformed hermaphrodites as seed parents in both populations for which I measured fruit production. Given that flowering females are nearly 20 times more likely to set fruit, it appears hermaphrodites are functioning primarily as pollen producers and the breeding system of *S. acaulis* may be closer to dioecy than gynodioecy. This is especially true for ES2, where hermaphrodites set close to no fruits, indicating their functional sex is closer to being male (Figure 1.6). Other studies also report low probabilities of setting any fruit by flowering hermaphrodites (Hermanutz and Innes 1994, Maurice *et al.* 1998).

Female advantage in the total quantity of fruits produced per plant also far exceeds the minimum advantage in seed fitness required for their persistence by models of nuclear or cytoplasmic sex determination (Lewis 1941, Lloyd 1975). These results agree with previous studies that have demonstrated a large female advantage for the proportion of flowers that set fruit in *S. acaulis* (Shykoff 1988, Hermanutx and Innes 1994), and Delph and Carroll (2001) further argue that fruit production is the primary source of female advantage across *S. acaulis* populations on Niwot Ridge, Colorado. Among studies that report the number of fruits produced per plant, the ratio of female fruits to hermaphrodite fruits is estimated at 4.4 in Alaska (Morris and Doak 1998) and from 9.27 for subspecies *cenisia* to 325 for subspecies *exscapa* in the French Alps (Maurice *et al.* 1998). Values from this study of 4.03 (BG1) and 27.46 (ES2) fall well within this range and suggest the existence of large variations among populations or possibly subspecies in hermaphrodite fruit production and hence their functional gender.

Given that gynodioecy is hypothesized to be a potential precursor to dioecy, and that subspecies within *S. acaulis* represent one of only two hypothesized evolutionary transitions from gynodioecy to dioecy within the genus *Silene* (Desfeux 1996), variation in the functional gender of hermaphrodites among populations and subspecies is of considerable interest in the study of gender evolution. In studies of *S. acaulis* where the subspecies designation is known, subsp. *exscapa* is observed to be functionally dioecious (Maurice *et al.* 1998), while subsp. *subacaulescens* clearly shows non-negligible fruit production by hermaphrodites and a gynodioecious breeding system (Shykoff 1988, Delph and Carroll 2001). In Alaska, Hultén (1968) describes both subsp. *exscapa* (= subsp. *acaulis*) and subsp. *subacaulescens*, though he notes they may introgress where their ranges overlap. Records show collections from similar geographic locations across Alaska for both subspecies (University of Alaska Herbarium), and morphological characters of plants grown in the common environment of the greenhouse suggest that both may be present in my study populations (S. Keller personal observation). However, inconsistent and intermediate morphologies in this study made absolute determination of subspecies based on morphological characters impossible. This could be attributable to introgression of subspecies *exscapa* with *subacaulescens* in some Alaskan populations, as suggested by Hultén (1968), and may have important implications for the functional gender of hermaphrodites and the breeding system of the population.

The observed association between female frequency and offspring quality lends support to the idea that obligate outcrossing by females increases the mean heterozygosity and related fitness traits in a population (Gouyon and Couvet 1987).

Increases in the proportion seed germination and decreases in seedling mortality were both observed in conjunction with increasing female frequency, though only germination was statistically significant (Figure 1.7). Studies of other *Silene* species have also found correlations between fitness traits and factors thought to affect genetic diversity such as the size and isolation of populations (Menges 1991, Richards 2000). However, in *T. vulgaris*, Tarayre and Thompson (1997) showed no relationship between female frequency and levels of allozyme heterozygosity. In the present study, because maternal sex is not known for the majority of seed parents, it is impossible to definitively conclude whether offspring from female and hermaphrodite mothers from a population with a given sex ratio show similar levels of seed viability. However, among the 15 individuals for which maternal sex was known, there were no differences between female and hermaphrodite mothers for proportion germination, a result that agrees with Shykoff (1988) and Keller (Chapter 2) who both showed no effect of maternal sex on proportion germination in controlled crosses. Therefore, in my study populations of *S. acaulis*, female biased populations outperform hermaphrodite biased populations in fruit production and offspring viability, a result that could be relevant to recent theory on group-level selection in gynodioecious plants and the evolution of the sex ratio (McCauley and Taylor 1997).

Summary

Alaskan populations of *S. acaulis* show considerable variability in the spatial distribution of females and hermaphrodites, with most of the variation distributed

between populations. Spatial patchiness of females within some populations indicates that the genes controlling sex expression may be finely structured, but this effect is modest overall in scale and frequency of occurrence. Heterogeneity among populations in sex ratio may represent differences in the diversity and equilibrium frequency of sex determining genes, as the majority of populations show no sign of nonequilibrium sex ratios as inferred from size class distributions. When the frequency of the sexes does differ across size classes, the trend is for fewer females among the smaller plants, indicating a decrease in female frequency over time that may be due to an increasing frequency of nuclear restorers following invasion by a CMS gene. Females far exceed hermaphrodites in fruit production, with the magnitude of the advantage more than compensating for their loss of male fitness. Additionally, the presence of females may actually benefit the population by enhancing the outcrossing rate and increasing progeny viability. Overall, the genetics of male sterility in *S. acaulis* appear to create spatially complex and variable patterns of sex expression that may have important implications for seed fitness in natural populations.

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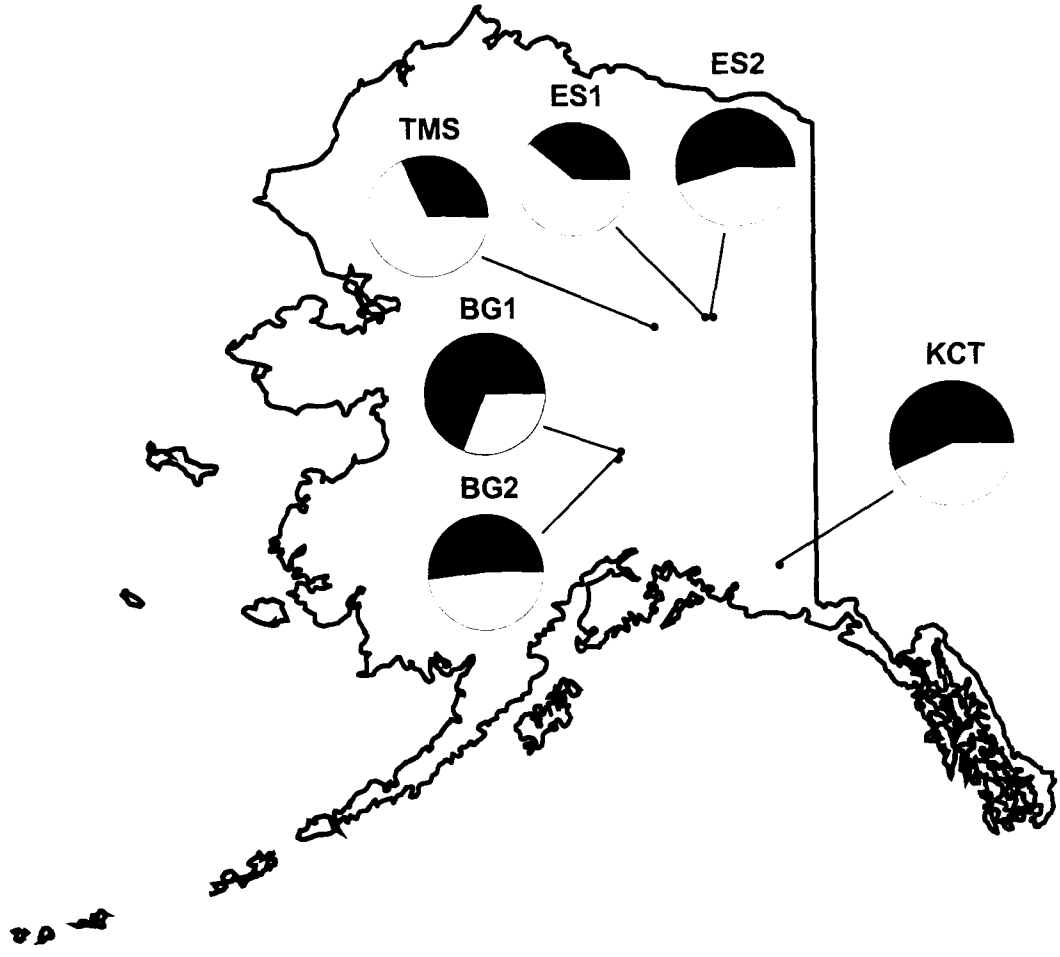

Figure 1.1. Map of Alaska, U.S.A. showing approximate location of 6 study populations. Pie diagrams represent the population sex ratio. Females are the shaded portions; hermaphrodites are the open portions.

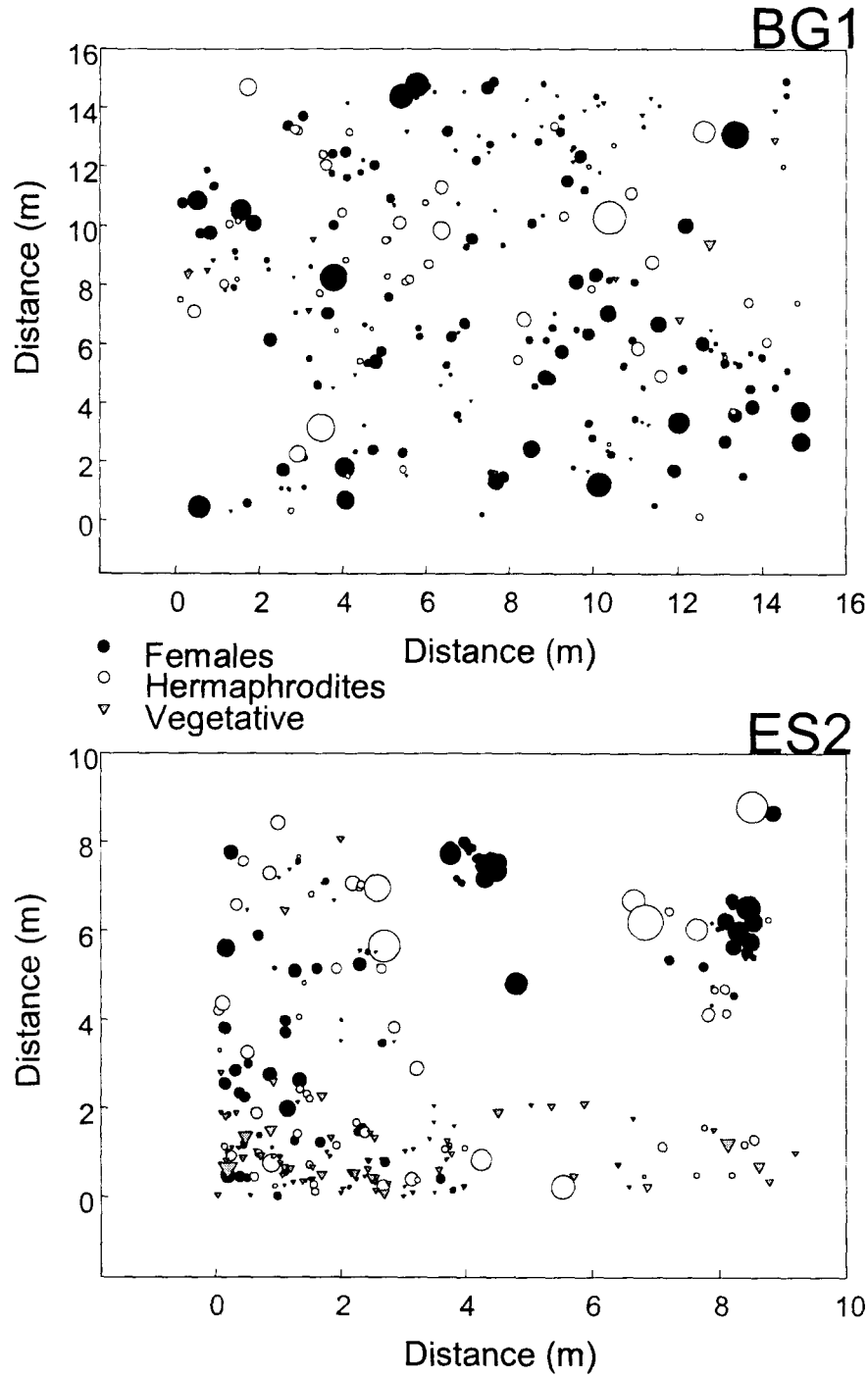

Figure 1.2. Maps of populations BG1 and ES2. Symbol size reflects the size diameter of each plant. Vegetatives = nonflowering plants.

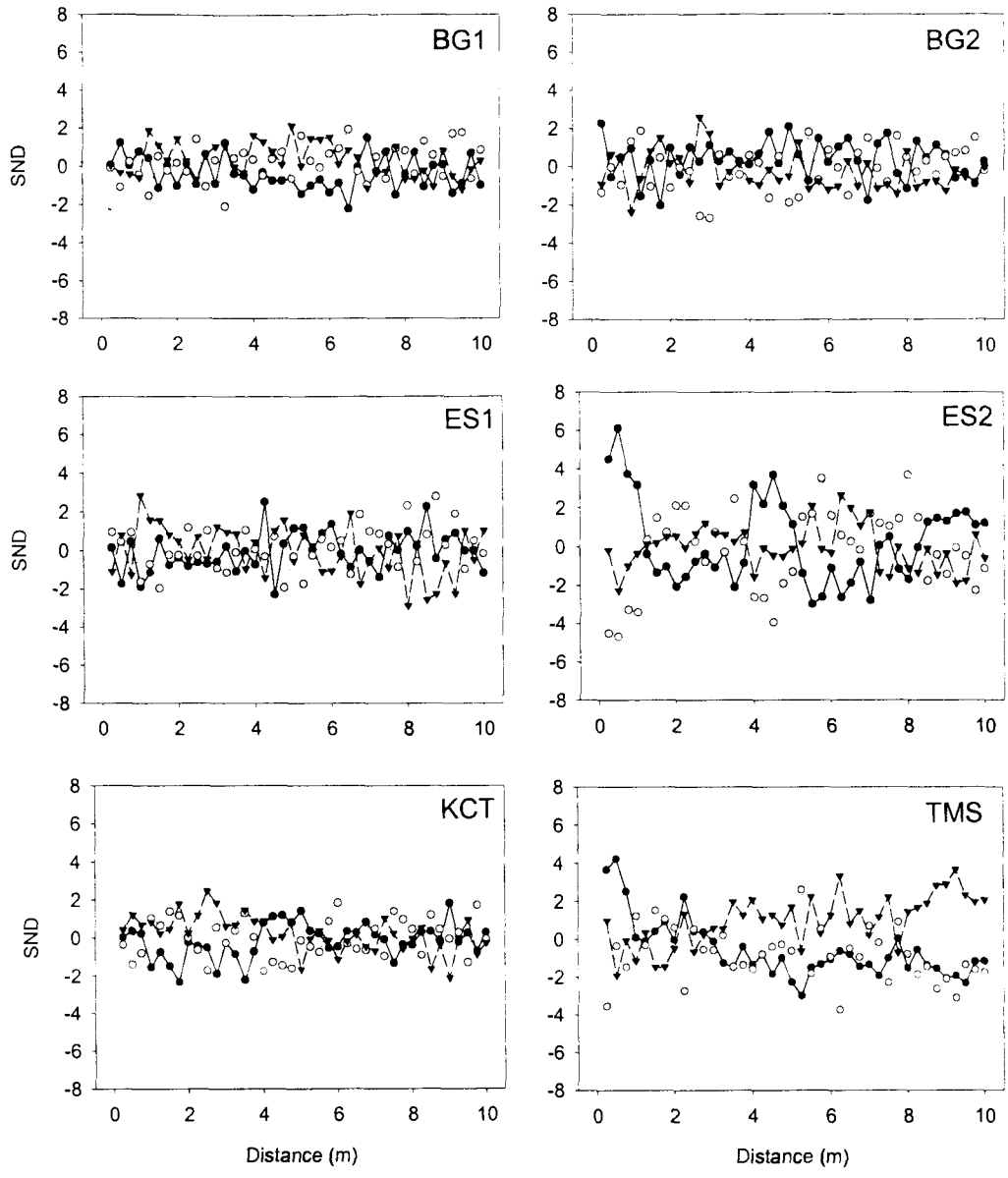

Figure 1.3. Correlograms for sex in six populations. Filled circles are female-female joins, open circles are female-hermaphrodite joins, filled triangles are hermaphrodite-hermaphrodite joins. Significant autocorrelation of sex at a distance interval is given by $|SND| \geq 2$.

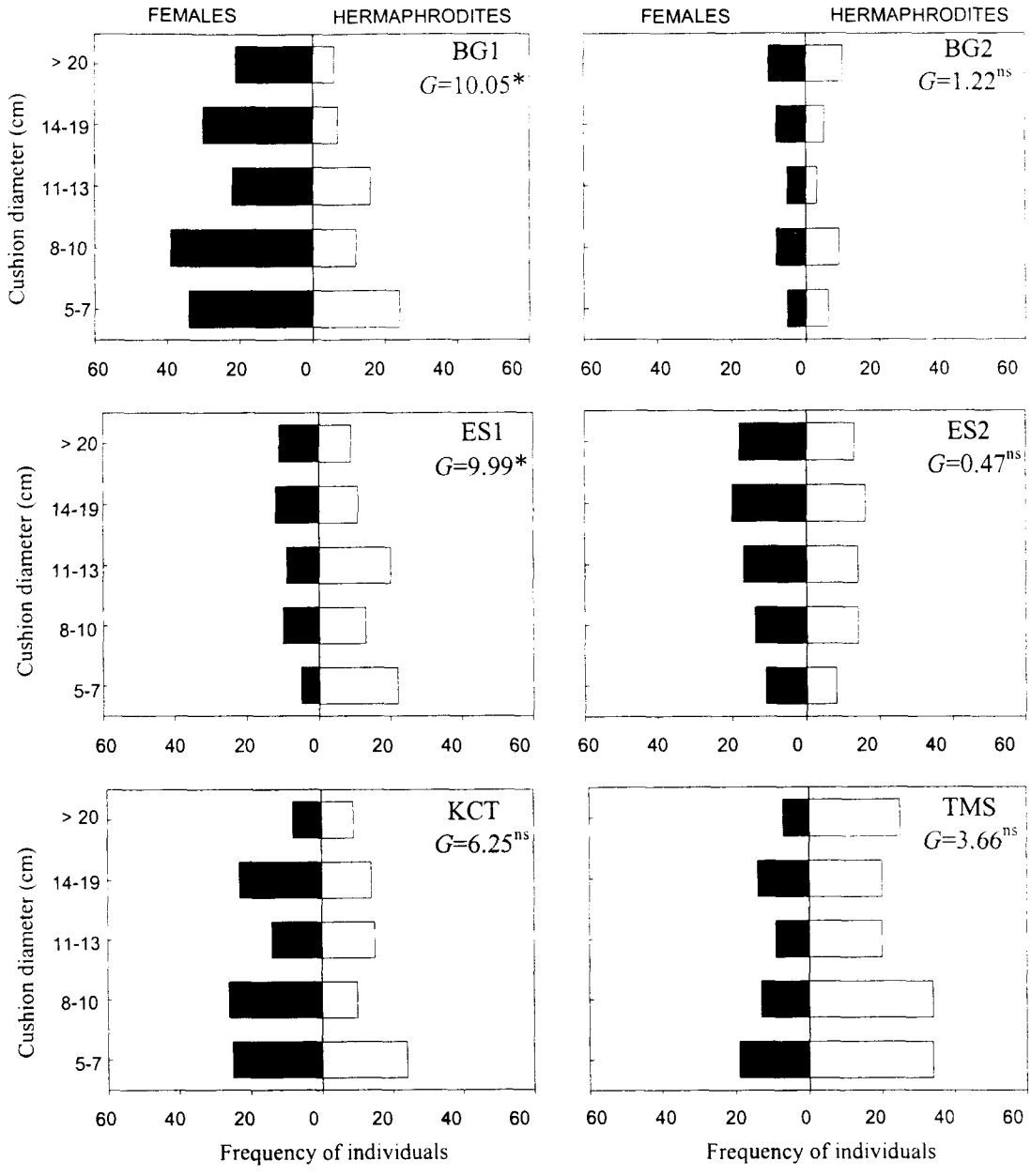

Figure 1.4. Frequency of the sexes across size classes for six populations. Females are shaded bars, hermaphrodites are the open bars. *G*-test of independence between sex and size, * $P < 0.05$; ns = nonsignificant.

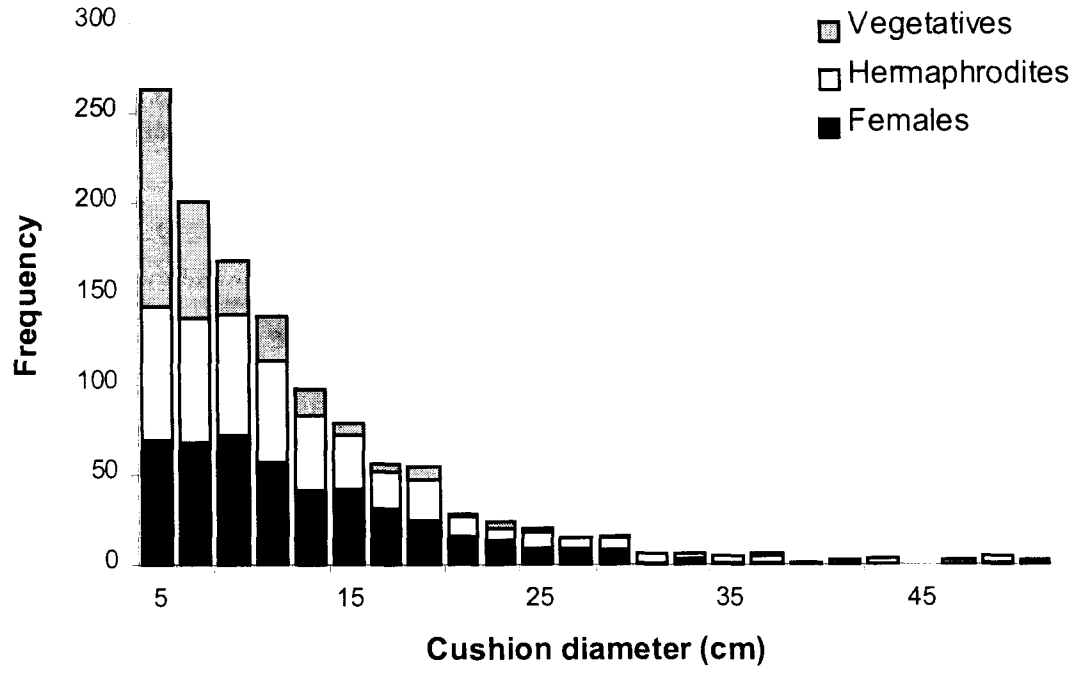

Figure 1.5. Size distribution of *Silene acaulis* cushions pooled across populations. Vegetatives = nonflowering plants during 2001.

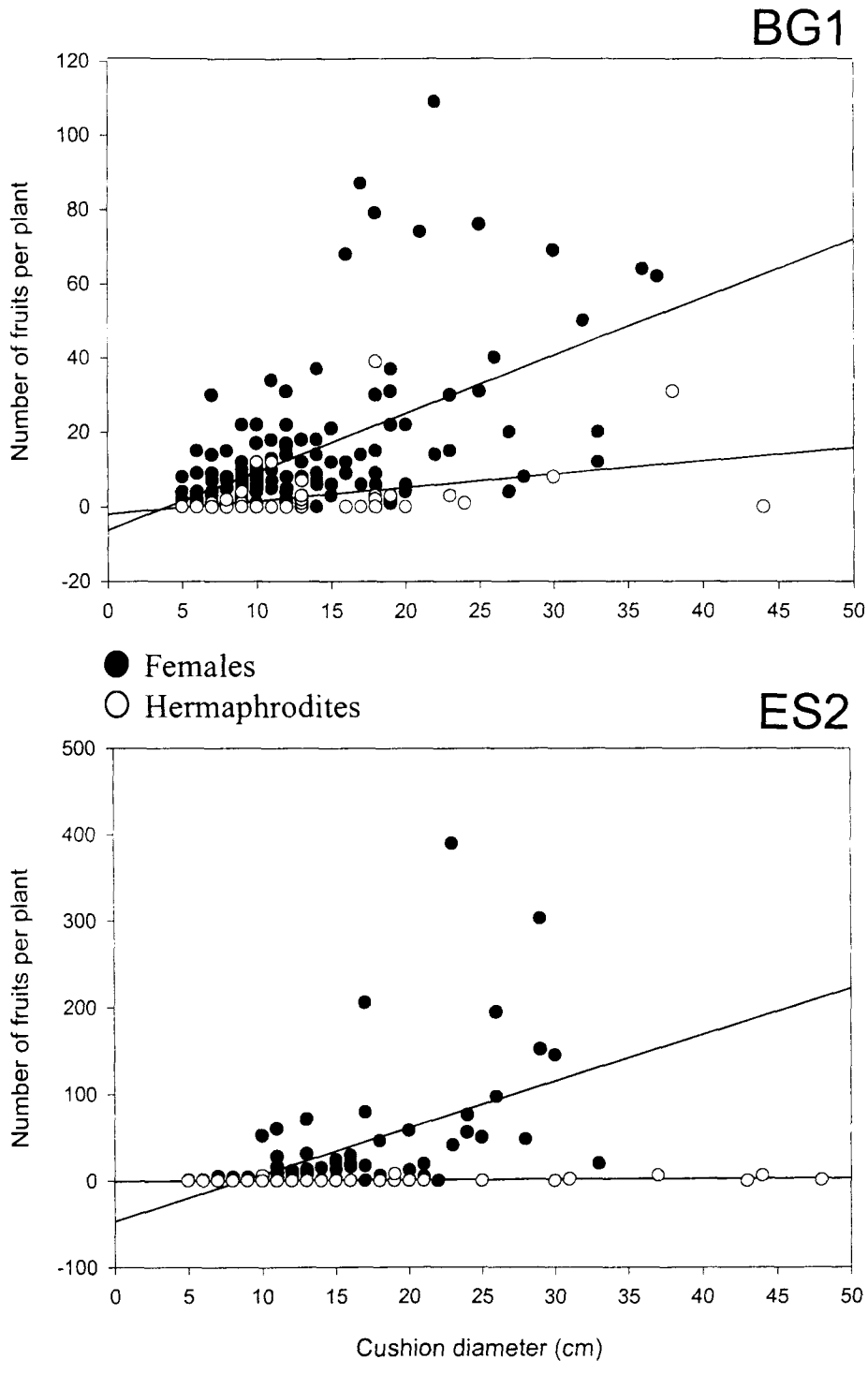

Figure 1.6. The relationship between cushion size and fruit production for females and hermaphrodites from populations BG1 and ES2 during 2001.

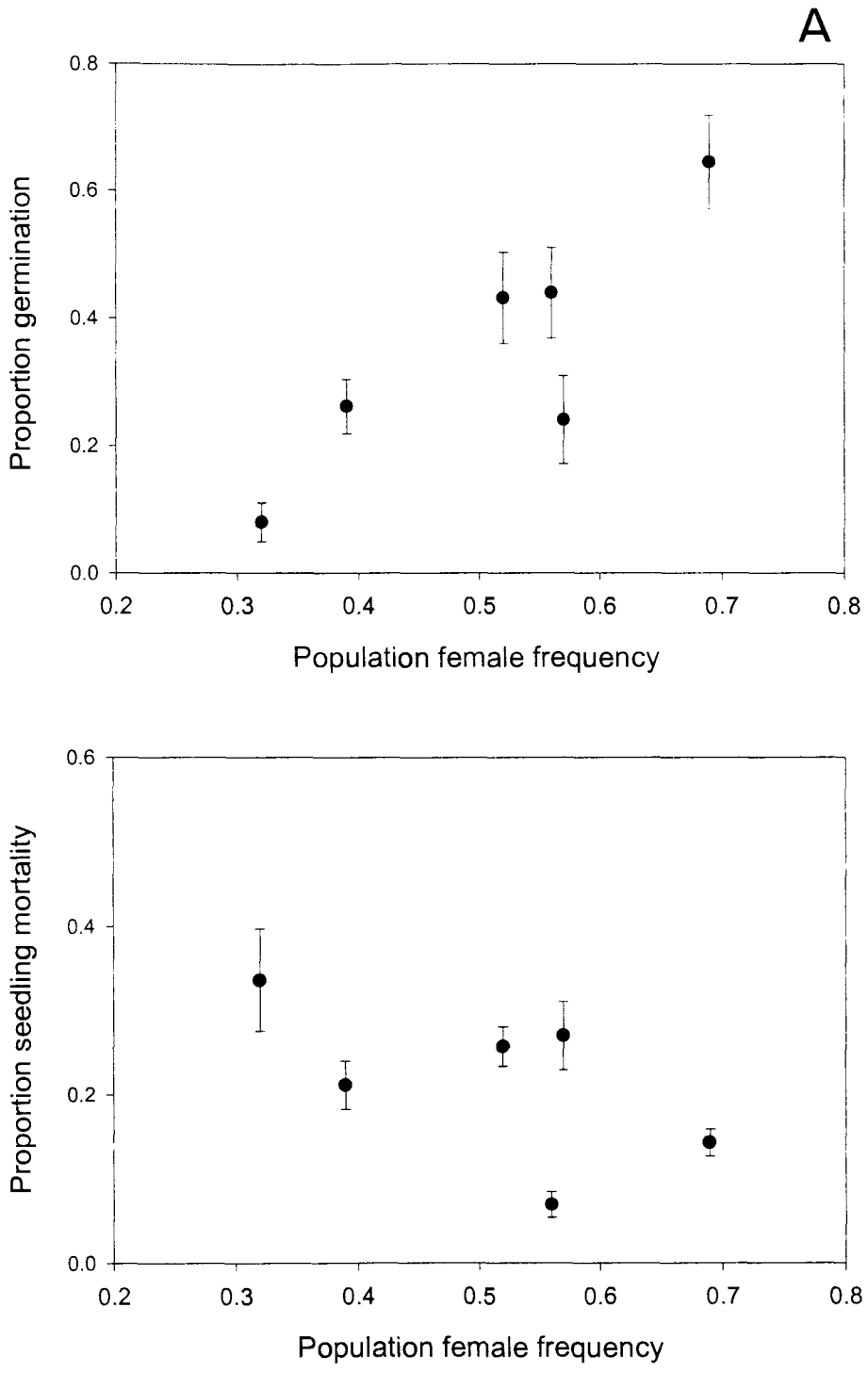

Figure 1.7. Female frequency and seed viability across six populations. A) Proportion germination; B) proportion seedling mortality. Data are population means (± 1 SE) of open-pollinated families.

Table 1.1. Characteristics of six Alaskan study populations of *Silene acaulis* sampled in 2001.

Population	Abbrev.	Latitude	Longitude	Plot size (m^2)	Number			Density (plants · m^{-2})	Female frequency [a]
					F	H	V		
Bison Gulch 1	BG1	N 63° 48′	W 148° 58′	100	146	65	34	2.45	0.69
Bison Gulch 2	BG2	N 63° 48′	W 148° 59′	300	36	33	8	0.26	0.52
Eagle Summit 1	ES1	N 65° 28′	W 145° 25′	300	47	75	90	0.55	0.39
Eagle Summit 2	ES2	N 65° 29′	W 145° 24′	90	80	65	92	2.63	0.55
Kennicott	KCT	N 61° 30′	W 142° 50′	105	96	72	32	1.90	0.57
Twelve Mile Summit	TMS	N 65° 24′	W 146° 01′	384	62	133	24	0.57	0.32

F = female, H = hermaphrodite, V = vegetative (non-flowering). [a] *G*-test of heterogeneity in female frequency among populations: $G = 69.34$, df = 5, $P < 0.0001$.

Table 1.2. Analysis of covariance for fruit production during 2001. Population (BG1 & ES2) and sex were fixed main effects; cushion size was the covariate. Model $R^2 = 0.56$.

Source	**df**	**MS**	***F***	***P***
Population (Pop)	1	9.970	10.27	0.0015
Sex	1	1.930	1.99	0.1597
Pop*Sex	1	6.400	6.60	0.0106
Size	1	132.270	136.30	<0.0001
Pop*Size	1	3.020	3.11	0.0787
Sex*Size	1	40.330	41.56	<0.0001
Pop*Sex*Size	1	10.410	10.73	0.0012
Error	339	0.971		

CHAPTER 2:

OFFSPRING QUALITY IN THE GYNODIOECIOUS PLANT *SILENE ACAULIS* (CARYOPHYLLACEAE): THE INDEPENDENT EFFECTS OF INBREEDING AND MATERNAL SEX[1]

INTRODUCTION

Understanding the factors driving the evolution of gender dimorphism is a central issue in evolutionary biology, and the diversity of breeding systems in higher plants makes them well suited for addressing this issue. Gynodioecy is a type of gender dimorphism characterized by the co-occurrence of both female and hermaphrodite individuals within populations (Darwin 1877). Next to hermaphroditism, gynodioecy is the second most widespread breeding system in angiosperms, comprising approximately 7% of all known species (Richards 1997). As an intermediate stage along the continuum from cosexuality to unisexuality, gynodioecy is an important study system for many theoretical and empirical investigations seeking to understand the factors affecting the evolution of gender dimorphism in plants (reviewed in Charlesworth 1999, Webb 1999).

One fundamental consequence of the loss of male function in females of gynodioecious species is the reduction in potential gene transmission compared to a hermaphrodite plant. This is because females contribute their genes only through ovules, whereas hermaphrodites can contribute genes through both ovules and pollen. Consequently, for male-sterile phenotypes to persist in a population, they must possess an

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advantage over hermaphrodites as seed parents (Lewis 1941, Lloyd 1974, 1975; Charlesworth and Ganders 1979). Achieving this advantage seems plausible because resources saved by not producing pollen may be allocated towards the production and provisioning of seeds by females (Poot 1997, Ashman 1999). The magnitude of the advantage necessary for the maintenance of females is dependent on the genes determining sex expression. If nuclear genes determine sex, females require at least a two-fold advantage in lifetime fitness as seed parents (Lewis 1941, Lloyd 1974,1975). If cytoplasmic genes are involved in sex expression (i.e., cytoplasmic male sterility genes, or CMS), as in many gynodioecious species, this condition is less restrictive, and females need only exceed hermaphrodites in seed fitness (Lewis 1941, Lloyd 1974,1975; Charlesworth and Ganders 1979).

Females may achieve a seed fitness advantage over hermaphrodites by making more seeds, seeds of higher quality, or both. Empirical studies often report female advantage in the quantity of fruit and seed produced (Shykoff 1988, Kohn 1989, Ågren and Wilson 1991, Delph and Lloyd 1991, Delph and Carroll 2001). Additionally, the expression of deleterious genes following inbreeding may affect offspring quality and is thought to be an important factor shaping the evolution of plant mating systems (Lande and Schemske 1985, Uyenoyama 1986, Charlesworth and Charlesworth 1987). Because the vast majority of gynodioecious species are self-compatible, self-fertilization by hermaphrodites may result in inbreeding depression in their seed offspring, whereas the ovules of females are obligately outcrossed. Lloyd (1975) first modeled this idea theoretically and showed that the selfing rate and inbreeding depression in hermaphrodite

mothers is proportional to the equilibrium frequency of females with nuclear or cytoplasmic modes of sex determination. In support of this prediction, Sun and Ganders (1986) showed that as the selfing rate of hermaphrodites increases among populations, so too does the frequency of females. However, without quantitative estimates of inbreeding depression, it can only be inferred that selfing rates coincide with reduced vigor in the inbred progeny of hermaphrodite mothers and a subsequent outcrossing advantage to females. Quantifying inbreeding depression as a source of female advantage in offspring quality is therefore a major goal for studies investigating the maintenance of females in gynodioecious species.

The relationship between offspring quality and inbreeding in gynodioecious species is complex. Poor performance of offspring from selfed hermaphrodites compared to females is commonly observed (e.g., Kesseli and Jain 1984, Pettersson 1992); however, this may arise due either to inbreeding depression or traits associated with the sex of the maternal parent. Females and hermaphrodites may differ in their maternal function through patterns of resource allocation and seed provisioning (Eckhart and Chapin 1997, Poot 1997), sexual selection (Wilson and Burley 1983, Shykoff 1992) or sex-linked genes with fitness effects (e.g., de Hann 1997a). Therefore, studies that compare selfed to outcrossed offspring of both hermaphrodites and females, thereby separating effects of inbreeding from maternal sex, are critical for understanding how maternal sex and inbreeding act independently in determining offspring performance (Shykoff 1988, Ashman 1992, Thompson and Tarayre 2000).

Further, both sexes are capable of biparental inbreeding. Biparental inbreeding is expected to occur within populations when spatial genetic structure and restricted pollen dispersal lead to mating among related individuals (Levin 1984, Uyenoyama 1986, Stacey 2001). Estimates of outcrossing rates and Wright's inbreeding coefficient (F_{IS}) indicate that biparental inbreeding may be a common feature in many populations of gynodioecious plants (Sun and Ganders 1988, Tarayre and Thompson 1997, Gehring and Delph 1999). If females of gynodioecious species routinely mate with relatives due to spatial genetic structure and local pollen flow, then the outcrossing advantage of females may be tempered by biparental inbreeding depression in their offspring. Therefore, the response of females and hermaphrodites to similar levels of biparental inbreeding may be important in determining the relative quality of their offspring and hence the outcome of selection on the sex polymorphism.

Several lines of reasoning suggest females and hermaphrodites may differ in their response to inbreeding. First, in self-compatible species, hermaphrodites may have a greater history of inbreeding compared to females because of their ability to self-fertilize. Second, neighborhood sex ratio bias may lead to greater biparental inbreeding in hermaphrodites versus females, as a patch of related hermaphrodite can freely mate with one another whereas a patch of related females must receive pollen from a more distant source. Such spatial sex structure is expected to develop when cytoplasmic genes lead to offspring sex ratios that are biased towards the sex of the mother and seed dispersal is localized (Gouyon and Couvet 1987, Kohn 1989, Manicacci *et al.* 1996, Graff 1999, Laporte *et al.* 2001). Through a combination of selfing and biparental inbreeding,

hermaphrodites may show reduced levels of inbreeding depression compared to females, as inbreeding lineages are expected to purge their genetic load of deleterious recessives compared to outcrossers (Lande and Schemske 1985, Husband and Schemske 1996; but see Byers and Waller 1999). Females on the other hand, should maintain a higher load of deleterious genes due to their inability to self, and with maternal bias in offspring sex ratios, from reduced biparental inbreeding caused by a deficit of related hermaphrodite pollen donors. Thus, the performance of their offspring may be exceeded by offspring from hermaphrodites under equivalent levels of biparental inbreeding.

Silene acaulis L. is a long-lived gynodioecious species with a Holarctic distribution. Field surveys have shown females have a large advantage in the quantity of fruits produced in the field relative to hermaphrodites (Shykoff 1988, Maurice *et al.* 1998, Morris and Doak 1998, Delph and Carroll 2001, Keller Chapter 1), and population sex ratios commonly vary from being hermaphrodite to female biased (Hermanutz and Innes 1994, Philipp 1997, Maurice *et al.* 1998, Keller Chapter 1). Strong inbreeding depression and maternal sex effects on offspring quality were reported for one population (Shykoff 1988). However, it is unclear to what extent inbreeding and maternal sex influence offspring quality across populations of gynodioecious species such as *S. acaulis*, which are likely to differ dramatically in their sex ratio (see references above), their history of inbreeding (Abbot *et al.* 1995, Phillip 1997, Gehring and Delph 1999), and probably in founder events and other demographic attributes likely to influence the effective population size (e.g., Benedict 1989, Philipp *et al.* 1990, Philipp 1997). To understand the ubiquity of inbreeding depression and maternal sex effects in causing

fitness differences between females and hermaphrodites as seed parents, and thus the evolution of gender dimorphism, more studies are needed that look at the effects of multiple levels of inbreeding independently on both sexes and in different populations.

In this chapter, I report the results of a greenhouse experiment designed to assess the independent and interactive effects of inbreeding and maternal sex on offspring quality across four Alaskan populations of *S. acaulis*. I address the following questions: (1) Do offspring produced by self-fertilized hermaphrodites differ in quality compared to outcrossed offspring? (2) Do biparentally inbred offspring differ in quality compared to outcrossed offspring? (3) Do offspring from females and hermaphrodites differ in their response to biparental inbreeding? (4) Are there differences between the sexes as seed parents, independent of inbreeding? (5) How do these effects differ among populations?

Materials and Methods

Study Species

Silene acaulis L. Jacq. (Caryophyllaceae) $2N = 24$, is a long-lived perennial distributed widely throughout arctic and alpine environments in North America and Europe. The breeding system in *S. acaulis* is highly variable, with populations reported as dioecious (Desfeux *et al.* 1996), trioecious (Hermanutz and Innes 1994, Philipp 1997, Maurice *et al.* 1998), and gynodioecious (Shykoff 1988, Morris and Doak 1998, Delph and Carroll 2001). Among studied populations in Alaska, hermaphrodites and females are both observed thus making the populations gynodioecious, although hermaphrodites generally have very low fruit set (Morris and Doak 1998, Keller Chapter 1). A small

proportion of gynomonoecious plants, producing both female and hermaphrodite flowers, are also present in Alaskan populations (Morris and Doak 1998, S. Keller personal observation). Two subspecies, subsp. *exscapa* (= subsp. *acaulis*) and subsp. *subaculescens*, have been reported throughout Alaska and are thought to transition into one another where their ranges overlap (Hultén 1968).

Individual plants grow as compact cushions of rosettes with single taproots and no adventitious rooting. Hermaphrodites are self-compatible and highly protandrous, with each flower dehiscing two whorls of five anthers prior to style elongation and female receptivity (Shykoff 1988, 1992). Females are male sterile and generally produce three long styles along with rudimentary anthers that fail to produce pollen. The flowers are strongly scented and pollinated primarily by bumblebees, although a variety of insect visitors are also commonly observed, including moths, butterflies, flies, beetles, and ants (Philipp *et al.* 1990, Delph and Carroll 2001, S. Keller personal observation). Females typically produce more fruits than hermaphrodites (Shykoff 1988, Hermanutz and Innes 1994, Maurice *et al.* 1998, Delph and Carroll 2001), with the ratio of female fruit production to hermaphrodite fruit production for studied populations in Alaska ranging from 4 to 27 (Morris and Doak 1998, Keller Chapter 1). The average number of seeds per fruit varies, but is generally below 20 (Shykoff 1988, Delph and Carroll 2001, Keller unpublished data). Seeds are small, generally weighing less than 0.5 mg (Shykoff 1988, Delph *et al.* 1999, this study), and possess no special dispersal mechanism. Several lines of evidence suggest gender in *S. acaulis* is determined by the joint influence of CMS genes and nuclear restorer genes. Maternal plants give rise to significantly biased sex

ratios in their offspring (Delph unpublished data cited in Delph and Carroll 2001, S. Keller unpublished data), populations vary widely in their sex ratio with female bias not uncommon, and experimental studies in a congener (*S. vulgaris*) have shown sex determination to be under cyto-nuclear control (Charlesworth and Laporte 1998, Taylor *et al.* 2001).

Greenhouse Experiment

Four study populations growing in alpine habitats in interior and southcentral Alaska were chosen for inclusion in an inbreeding experiment (Table 2.1). Populations were located in one of three distinct mountain ranges in Alaska: the White Mountains (ES2), the Alaska Range (BG1 and BG2), and the Wrangell Mountains (KCT). Populations were separated by geographical distances of 0.4 – 460.0 km and span 4° of latitude (Table 2.1). During late August 2000, I selected 11 to 17 fruiting plants along walking transects of each population and collected five mature fruits from each individual, together representing a single maternal sibship. Plants were selected at a minimum distance of three meters apart to minimize relatedness in the sample resulting from any spatial genetic structure (e.g., Gehring and Delph 1999, Keller Chapter 1). Fruits were placed in cold storage at 4° C for approximately four months. Beginning in January 2001, I germinated seeds (2511 total from 60 maternal sibships) and grew plants in the greenhouse under a 24 hr photoperiod. Plants were watered and fertilized on a regular basis. As plants flowered, I scored their sex as female if all flowers were pistillate, or as hermaphrodite if plants produced either all perfect flowers or a

combination of pistillate and perfect flowers. Since individuals producing both pistillate and perfect flowers are capable of producing pollen and seeds, as well as self-fertilizing, they are functionally hermaphrodite and were not analyzed separately.

I assigned female and hermaphrodite maternal plants to one of four pollination treatments reflecting different inbreeding levels: (1) hermaphrodites received self pollen from another flower in male phase on the same plant (geitonogamy), corresponding to an offspring inbreeding coefficient of 0.5; (2) hermaphrodites and females received pollen from a related hermaphrodite belonging to the same maternal sibship and population, with an inbreeding coefficient between 0.125 and 0.25; (3) hermaphrodites and females received outcross pollen from an unrelated hermaphrodite belonging to a different maternal sibship from the same population, with an approximate inbreeding coefficient of 0; (4) hermaphrodites and females received outcross pollen from an unrelated hermaphrodite originating from a different population, with an inbreeding coefficient of 0. Because field collected maternal sibships were open pollinated and of unknown paternal parentage, they may consist of a mixture of full and half sibs. I included between-population outcrosses to serve as an additional outcrossed standard against which to evaluate the fitness of inbred crosses. By assigning individuals to only one inbreeding treatment level, I controlled for any within-plant bias in resource provisioning to developing seeds with different inbreeding levels (Wilson and Burley 1983, Ashman 1992).

I performed hand pollinations of open flowers by brushing pollen from freshly dehisced anthers across the receptive stigmatic surface of a flowering maternal plant. On

a given day, I selected a flowering hermaphrodite as a pollen donor if it had pollen-bearing flowers and satisfied the required level of relatedness between parents defined by the above treatments. Individual flowers on a maternal plant received pollen from only one donor, but different flowers on a single maternal plant often received pollen from different donors. Likewise, some pollen donors were mated to more than one maternal plant that potentially differed in their treatment levels. Hermaphrodite maternal plants in the sib and both outcross pollination groups were emasculated before pollination to minimize any possibility of selfing. To prevent uncontrolled pollination due to accidental pollen movement within the greenhouse, I inverted a 2 ml clear plastic microcentrifuge tube over each flower following pollination, and held it in place with a straight pin pushed into the cushion, as described by Delph *et al.* (1999). I performed pollinations from March to July 2001 for a total of 1874 individual flowers pollinated. Because of large variation in the germination and survival of individuals from each source population that comprised the parental generation, coupled with non-sequential flowering and a complex experimental design, the number of maternal plants pollinated from each population was highly variable (BG1: 189, BG2: 129, ES2: 88, KCT: 51). Each maternal plant was pollinated on an average of four of its flowers.

After a flower had senesced and if a fruit had begun to grow, I removed the microcentrifuge tube and later harvested the mature fruit when it turned brown, but generally prior to dehiscence. I counted the total number of filled seeds in each fruit and weighed all seeds individually to the nearest 0.001 mg on a Sartorius Micro electronic balance. Seeds were placed in individual glassine envelopes and stored at 4° C for

several months prior to planting. A total of 3757 seeds from 407 fruits were collected from 202 maternal plants. Each maternal plant was represented by a mean (SE) of 19.66 (1.71) seeds.

Seeds were planted in a completely randomized design among six trays filled with medium-fine vermiculite loosely packed into 1 cm^3 plastic lighting grid. Trays were misted with water and placed into a Conviron growth chamber set to 16° C and a 19:5 hr (L:D) photoperiod. Seeds were misted and observed for germination daily for four weeks, after which they were checked every two days until a total of six weeks had passed. After six weeks, germination had largely ceased, and I considered ungerminated seeds unviable. Upon germinating, each seedling was transferred by hand to an individually labeled 30 ml plastic pot filled with wetted vermiculite, watered with 5 ml dilute liquid fertilizer, and placed in a numbered flat into one of two additional growth chambers under identical environmental conditions. Each flat of seedlings was rotated in the growth chambers on a daily basis to reduce position effects. Plants were misted with water daily and seedlings were individually scored for mortality every seven days following the day they germinated. If a seedling was still alive at the end of each seven day period, it received an additional 5 ml of fertilizer and remained in the growth chamber for another week. Three weeks after planting, seedlings were removed from pots, roots were washed free of vermiculite, and the entire seedling was dried to constant mass at 70° C. The dry mass of each seedling (1510 total from the 131 maternal plants still represented at harvest) was determined to the nearest 0.01 mg on a Mettler AE 240 electronic balance. A multiplicative measure of the cumulative offspring quality

produced by each maternal plant was formulated as proportion germination * (1-proportion mortality) * mean seedling dry mass (mg).

Statistical Analyses

The experimental design consisted of a three-way treatment structure of population, sex, and inbreeding treatment and was analyzed using analysis of variance (ANOVA; PROC GLM: SAS Institute 1988). In order to better conform to ANOVA assumptions of normality and homoscedasticity, variables were either natural-log transformed (seed mass, days to germination, seedling dry mass, cumulative offspring quality) or arcsine-square root transformed (proportion germination, proportion mortality) prior to analysis. Because the experimental design contains a structurally empty cell (females are incapable of self-fertilization), I combined sex and inbreeding treatment into a single explanatory variable (cross type) with 7 levels (Figure 2.1). This approach accommodates direct comparisons among all the levels of maternal sex and inbreeding treatment, with the inclusion of selfs, in a complete design. Therefore, I employed a two-factor ANOVA to test differences among populations, among cross types, and the interaction between these effects. For the variables seed mass, days to germination, and seedling dry mass, maternal plant was included as a random effect nested within population and cross type. Main and interaction effects were tested with the maternal plant mean square as the denominator in the *F*-test (Zar 1999 App 8). I also included a blocking factor for error control in the analysis of days to germination (block = germination tray) and seedling dry mass (block = growth chamber). The remaining

variables, proportion germination, proportion mortality, and cumulative offspring quality, are properties of maternal plants, so the analysis was a two-way fixed effects model with *F*-tests calculated using the mean square error in the denominator. To help control for variability in offspring performance contributed by unintended maternal effects (environmental or genetic), I included the mass of each individual seed as a covariate when analyzing days to germination and seedling dry mass, and mean seed mass of each maternal plant when analyzing proportion germination, proportion mortality, and cumulative offspring quality.

To investigate differences among levels of the population main effect, I compared least squares means using Tukey-Kramer post-hoc comparisons. If the cross type effect was significant ($P < 0.05$), I used *a priori* planned contrasts to test the following hypotheses (PROC GLM CONTRAST statement, Table 2.2): 1) no difference between selfs and within-population outcrosses, 2) no difference between selfs and between-population outcrosses, 3) no difference between sib crosses and within-population outcrosses, 4) no difference between sib crosses and between-population outcrosses, 5) no difference among within-population and between-population outcrosses, 6) no difference between females and hermaphrodites (not including selfs), and 7) no sex by breeding treatment interaction. The sex by breeding treatment interaction effect was tested using three contrasts that span the interaction space in the design (Table 2). These three interaction contrasts tested the equality of the response of the sexes to a) sib crosses vs. between-population outcrosses, b) sib crosses vs. within-population outcrosses, and c) within-population vs. between-population outcrosses. For the variables seed mass, days

to germination, and seedling dry mass, significance tests for contrasts were constructed using the maternal plant mean square in the denominator, as per the overall cross type effect. Contrasts for all other variables used the mean square error in the denominator for significance tests.

To estimate the relative fitness of inbred versus outcrossed offspring, I calculated inbreeding depression separately for each inbred maternal plant as $\delta = 1 - W_I / W_{BPOX}$, where W_I is the cumulative offspring quality produced by inbred maternal plants (self, sib, or within-population crosses) and W_{BPOX} is the mean offspring quality of between-population outcrossed plants, specific to each maternal sex and source population. Thus, each inbred family is evaluated relative to the between-population outcross mean for the sex and population to which it belongs. According to this formulation, δ values > 0 indicate a reduction in fitness following inbreeding, while δ values < 0 indicate an enhancement of fitness following inbreeding. I chose to use the between-population treatment instead of the within-population treatment as the outcross standard because random crosses within a population may still be among related individuals. Crossing individuals from separate populations controls for this, although crosses between populations may also result in other genetic phenomena that affect fitness, such as the disruption of co-adapted gene complexes. To address this issue, I also compared within-population outcrosses to between-population outcrosses and evaluated whether the relative quality of the offspring from these two treatments was similar.

RESULTS

The mass of individual seeds varied among populations, but not among the different cross types (Table 2.3). The mean (± SE) seed mass from population BG1 was 0.270 ± 0.005 and significantly greater than all other populations, which did not differ from each other in seed mass (Tukey-Kramer pairwise comparisons; means ± SE, BG2: 0.213 ± 0.005, ES2: 0.233 ± 0.008, KCT: 0.228 ± 0.008). Hermaphrodite mothers produced seeds of comparable mass to females (means ± SE [N], hermaphrodites: 0.283 ± 0.004 [1223]; females: 0.272 ± 0.002 [2534]; Figure 2.2). There was no interaction between population and cross type for seed mass, although individual maternal plants nested within population and cross type displayed significant variation (Table 2.3).

There were no differences among cross types in the proportion of seeds that germinated ($F_{6, 201} = 1.34$, $P = 0.24$) or in the number of days to germination ($F_{6, 137} = 0.11$, $P = 0.99$), nor any interaction of cross type with population for either of these variables (proportion germination: $F_{18, 201} = 0.41$, $P = 0.98$; days to germination: $F_{18, 137} = 1.33$, $P = 0.17$). However, strong differences among populations existed in germination traits, even after controlling for the effects of seed mass (proportion germination: $F_{3, 201} = 3.92$, $P = 0.0097$; days to germination: $F_{3, 137} = 6.81$, $P = 0.0003$). Population KCT germinated a significantly smaller proportion of its seeds compared to BG1 and BG2, but did not differ in proportion germination from ES2 (Figure 2.3). Seeds from KCT also took significantly longer to germinate compared to the other three populations, although ES2 also showed a delay in germination compared to BG1 (Figure 2.3). Seed mass had a positive effect on germination traits, with maternal plants that

produced a large mean seed mass also having a greater proportion of seeds germinate ($F_{1, 201} = 38.98$, $P = <0.0001$), and individuals with larger seeds taking slightly longer to germinate ($F_{1, 1722} = 4.42$, $P = 0.0358$).

In contrast to germination traits, seedling mortality was uniform among the populations but varied among cross types (Table 2.4). Contrasts showed that this was due to high levels of seedling mortality among the progeny derived from the self-pollinated group compared to both the within-population and between-population outcrosses (Table 2.4). No effect of sib crossing or maternal sex was observed on seedling mortality, nor was there significant interaction among the sexes in their response to the different pollination treatments (Table 2.4; Figure 2.4). Seedling mortality showed no interaction between population and cross type. Seed mass once again had a large effect, with seedlings that germinated from families of smaller seeds more likely to die within the first three weeks of growth compared to seedlings from families with a greater mean seed mass (Table 2.4).

After three weeks of growth, significant differences in seedling dry mass existed among cross types. Contrasts revealed that offspring from self-pollinations had significantly less mass than offspring from between-population outcrosses (Table 2.5). No effect of sib crossing on dry mass was detected (Figure 2.5). There was a significant effect of maternal sex on offspring dry mass at harvest, with female mothers producing offspring with more plant mass than those from hermaphrodite mothers, after controlling for seed mass (Table 2.5), indicating that offspring from females exhibit higher growth rates when compared to offspring from hermaphrodites.

Cumulative offspring quality was significantly lower for selfed offspring than offspring from within- or between-population outcrosses (Table 2.6; Figure 2.6). Offspring from sib crosses were not different than offspring from outcrosses, nor were there any significant interactions between maternal sex and pollination treatment. Populations showed marginal differences, with mothers from BG1 tending to produce overall higher quality progeny (mean ± SE, 0.850 ± 0.395) than those from KCT (mean ± SE, 0.632 ± 0.402; Table 6). There was no significant interaction between populations and cross type (Table 2.6). Seed mass was an important contributor to offspring quality, with mother plants producing heavier seeds also having greater proportion germination, lower seedling mortality, and more seedling dry mass by the end of the experiment (Table 2.6).

Inbreeding depression in selfed offspring as a measure of relative offspring quality varied from 0.47 to 0.83 among the populations, with a mean among populations of 0.67 (Table 2.7). Population mean values of sib crossed hermaphrodites were variable, with δ ranging from -1.05 to 0.09. In contrast, females showed all positive values of δ, with population means ranging from 0.13 – 0.62. Pooled across populations, the ranked distribution of inbreeding depression based on each maternal plant shows a consistent reduction in relative offspring quality among selfed hermaphrodites, a trend towards reduced relative quality among sib crossed females, and a trend towards enhancement of relative quality among sib crossed hermaphrodites (Figure 2.7).

DISCUSSION

Inbreeding Effects on Offspring Quality

The reduced survival, growth, and cumulative quality of selfed offspring in this experiment all suggest appreciable genetic load exists within my study populations. This confirms the prediction that the avoidance of inbreeding depression can contribute to female advantage in gynodioecious species (e.g., Lloyd 1975). My estimates of inbreeding depression following selfing are intermediate when compared with the broad range of inbreeding depression values observed in other gynodioecious species, where δ ranged from –0.13 to 0.95 (13 studies reviewed in Mutakainen and Delph 1998). Combined, my data and the previously reported results suggest that considerable variation exists in the response of gynodioecious taxa to inbreeding, not only among species but also among populations within a species.

The inbreeding depression I observed may contribute to the equilibrium frequency of females in populations of *S. acaulis* if hermaphrodites regularly self-fertilize in the field. Given a mean δ among populations of 0.67 for selfing, an approximate ratio of female:hermaphrodite seed production of 4.0 for Alaskan populations (Morris and Doak 1998, Keller Chapter 1), and a selfing rate of approximately 0.30 for hermaphrodites (Marr 1997 cited in Gehring and Delph 1999), the frequency of females at equilibrium predicted by Lloyd's (1975) model is 0.31 for nuclear determination of sex and 0.89 for cytoplasmic determination. Since sex expression is thought to have a cytonuclear basis in *S. acaulis*, these two values set an upper and lower bound, and the actual equilibrium frequency should occur somewhere between these estimates. Female frequency in my

study populations varies from 0.52 to 0.69 (Table 2.1), and although the overall test is crude, they fall well within the range predicted by the model. Interestingly, given the above values for female advantage in fruit production and inbreeding depression in hermaphrodites, changing the selfing rate contributes little to shifting the equilibrium female frequency for either mode of sex inheritance (a maximum change in female frequency of 0.12 for nuclear and 0.09 for cytoplasmic sex determination). The effect of selfing is even further diminished if female advantage in fruit production is increased, as some populations of *S. acaulis* show values as high as 27 (Keller Chapter 1) and even 325 (Maurice *et al.* 1998). Fruit production advantage of this magnitude approaches infinity in the model and essentially results in dioecy, with the effects of selfing negated by the extremely few seeds produced by hermaphrodites that could potentially express inbreeding depression.

The reduction in offspring quality following self-pollination of hermaphrodites corroborates previous work on inbreeding in *S. acaulis* (Shykoff 1988), although differences are evident for the magnitude and timing of inbreeding depression between our studies. I found no effect of inbreeding on germination traits but significantly elevated mortality of selfed offspring compared to outcrosses, in agreement with Shykoff (1988). However, the mean mortality of selfed offspring that I observed was substantially lower than Shykoff (1988) obtained (53% vs. 99%). In addition, the reduced dry mass of selfs in my study contrasts with Shykoff (1988) who found no differences in seedling growth rate during the first three weeks following germination. This suggests the amount of genetic load and the life stages where it is manifested shows

variation among populations and/or experimental conditions. Offspring from both studies were greenhouse raised under near-optimal conditions. However, I grew seedlings individually while Shykoff (1988) grew seedlings collectively. Therefore, slow growing genotypes in my study may have persisted due to the absence of competition while competitively inferior genotypes in Shykoff's (1988) study were more likely to die. Moreover, it should be stressed that my results provide a minimum estimate of inbreeding depression in these four populations of *S. acaulis,* given that the establishment conditions were benign and observations limited to early life stages. Other investigators have observed minimal and episodic recruitment of *S. acaulis* in the field (Philipp et al. 1990), suggesting field conditions are strenuous and the reduction in fitness of selfed offspring may be much greater than in the greenhouse, as well as potentially compounded across a plant's lifetime.

Theory suggests differences in sex ratio can reflect both the outcome of selection on female advantage (Lewis 1941, Lloyd 1974, 1975) as well as the positive contribution of females to the outcrossing rate and to levels of heterozygosity (Gouyon and Couvet 1987). Thus, the magnitude of inbreeding depression observed may relate to the sex ratio of a population. Genetic models predict that when inbreeding depression is due to a few deleterious recessive genes with large fitness effects, outcrossing populations should maintain more genetic load and thus express greater inbreeding depression compared to selfing populations that may purge their load (Charlesworth and Charlesworth 1987, Husband and Schemske 1996). Alternatively, if inbreeding depression is due to overdominance of alleles at fitness loci or to many deleterious recessive genes each of

minor effect, then purging will be inefficient and selfing populations may actually exhibit greater inbreeding depression compared to outcrossing ones (Charlesworth and Charlesworth 1987, Schultz and Ganders 1996). In the latter case, a trait that positively affects the outcrossing rate of a population, such as male sterility, may result in less genetic load by preventing the buildup of loci homozygous for mildly deleterious alleles that results from inbreeding. The presence of many recessive deleterious alleles of minor effect may help explain why the female biased populations (with presumably higher outcrossing rates) I studied show less inbreeding depression compared to Shykoff's (1988) hermaphrodite biased population (with presumably lower outcrossing rates). However, in a test of the hypothesis that hermaphrodites and females should differ in their inbreeding history and thus their genetic load and expression of inbreeding depression, Mutakainen and Delph (1998) compared inbreeding depression in *Lobelia siphilitica* among parents derived from a female line and a hermaphrodite line and found no differences attributable to lineage. Clearly, understanding the relationship between inbreeding history, genetic load, and the sex ratio in gynodioecious populations awaits further experimental tests among many populations of different taxa.

In contrast to selfing, sib crossing had relatively slight and somewhat variable effects in this study (Table 2.7; Figure 2.7). There was a tendency towards values of $\delta > 0$ for most sib crossed females, while the majority of sib crossed hermaphrodites had values of $\delta < 0$, suggesting enhanced fitness following intermediate levels of inbreeding. This partially supports the prediction that females should suffer more from biparental inbreeding compared to hermaphrodites, although the distribution of hermaphrodite

values is surprising. Negative estimates of inbreeding depression in my study may simply reflect noise caused by a limited sample size, especially since none of the individual analyses of fitness traits showed significant differences for sib crossed versus outcrossed offspring, nor any significant interactions between sex and pollination treatment. However, if the trend towards enhancement of relative fitness in sib crossed hermaphrodites shown in Figure 2.7 is biologically meaningful, it could indicate the disruption of favorable interactions between the nuclear and cytoplasmic genomes in outcrossed hermaphrodites. For example, in *Phacelia dubia,* del Castillo (1998) found reduced fitness of sib crossed ($F = 0.25$) hermaphrodites for some traits (seeds per flower, survival to reproduction), and noticeably increased fitness for others (offspring seed production, percentage full pollen grains). He proposed that variable and nonlinear relationships between inbreeding and fitness might in part be attributable to cytonuclear interactions in gynodioecious species (del Castillo 1998). Other studies on non-gynodioecious plants and animals have also found increased fitness among sib crosses, for example in the hermaphroditic *Mimulus guttatus*, Willis (1993) found an increase in fitness following crosses among sibs ($F = 0.25$) for the probability of germination, and in flour beetles, Pray and Goodnight (1995) found enhanced fitness at intermediate levels of inbreeding for several developmental and fitness related traits. Together, these results suggest that fitness may not always decrease monotonically with inbreeding, and the genetic basis of inbreeding depression as well as the possibility of intergenomic epistasis (i.e., cytonuclear interactions) may create a complex relationship between inbreeding and fitness.

Other studies investigating sibling level inbreeding in gynodioecious plants report mixed results. In *Lobelia siphilitica*, Mutakainen and Delph (1998) found no consistent evidence for biparental inbreeding depression, although large differences in fitness existed among families. In a multi-population study of *Thymus vulgaris,* Thompson and Tarayre (2000) found inbreeding depression for sib crossed female and hermaphrodite mothers, although this varied among populations. Ashman (1992) found reduced progeny performance of sib crossed females, but less so for hermaphrodite mothers. In the present study, the overall influence of sib crossing on female advantage in *S. acaulis* appears weak or nonexistent, and is most likely overshadowed by advantages in fruit production in females and their avoidance of selfing.

Maternal Sex Effects on Offspring Quality

Prior investigations into the breeding system of *S. acaulis* have demonstrated that hermaphrodites mature significantly fewer fruits compared to females, potentially due to resource limitation (Shykoff 1988, Hermanutz and Innes 1994, Maurice *et al.* 1998, Morris and Doak 1998, Delph and Carroll 2001, Keller Chapter 1); however, hermaphrodites may also be limited in their ability to provision those seeds that they do develop. I observed no difference in seed mass between the cross types, suggesting that hermaphrodites are not resource limited in the quantity of provisions they allocate to individual seeds within the greenhouse environment (Figure 2.2). This result agrees with a previous study by Delph *et al.* (1999), who obtained hand outcrossed seeds of *S. acaulis* from the field and found either no difference or nonsignificantly higher provisioning by

hermaphrodite mothers compared to females for several measures of seed provisioning. The consistently strong effects of seed mass on fitness traits observed in *S. acaulis,* as well those reported in studies of other species (reviewed in Roach and Wulff 1987), highlight the importance of this trait on early stages of offspring development. It also implies selection will be very strong on traits affecting the provisioning of developing seeds in *S. acaulis,* although the evidence presented above suggests this doesn't directly involve maternal sex.

At the latest stage of offspring development observed, females produced seedlings with greater dry mass than hermaphrodites. This difference in offspring quality was detected after controlling for the effects of initial seed size, suggesting that offspring from females grew more vigorously than those from hermaphrodites. This relatively weak maternal sex effect contrasts with the strong effect Shykoff (1988) observed on survivorship, where a greater proportion of seedlings from outcrossed females survived compared to those from outcrossed hermaphrodites. Delph *et al.* (1999) proposed that negative pleiotropic effects of restorer alleles carried by hermaphrodite mothers and transmitted to their offspring may explain the differential mortality among offspring of females and hermaphrodites that Shykoff (1988) observed. The concept of a "cost of restoration" was first introduced as a variable in theoretical models, and defined as a reduction in fitness that results from a mismatch between a CMS type and a restorer at a locus that is not specific to that CMS type (Frank 1989, Gouyon *et al.* 1991). In the sense that CMS represents a sterilizing "disease" and restorers the "cure", the cost of restoration in gynodioecy is analogous to the cost of resistance often shown in host-

pathogen relationships (e.g., Biere and Antonovics 1996). Despite the importance of the cost of restoration in recent models of gynodioecy, experimental tests of its existence in natural populations are rare (but see de Hann *et al.* 1997a). Theoretically, if the population sex ratio in the field reflects the current frequency and diversity of male fertility restorers (de Hann *et al.* 1997b, Manicacci *et al.* 1997), then the sex ratio should covary with any observed cost of restoration. All my study populations show female biased sex ratios (Table 2.1), presumably due to low availability of appropriate restorers. In contrast, Shykoff's (1988) population contained only 25% females, indicating that restoration is much more frequent and associated costs of carrying mismatched restorers may be responsible for the large maternal sex effect on survivorship. A first step towards elucidating whether sex ratio is predictive of the cost of restoration would be to correlate the presence and magnitude of maternal sex effects with estimates of the rate of restoration in populations that span a gradient of sex ratios.

Experimental studies of gynodioecy often differ from one another in the importance of maternal sex in determining progeny quality. For example, among studies that found a significant maternal sex effect on offspring quality after controlling for cross type, seeds from female mothers showed either a greater likelihood of germinating (Ashman 1992), a slightly lower likelihood of germinating (Jolls and Chenier 1989), higher seedling survivorship (Shykoff 1988), or higher juvenile growth rates when compared to hermaphrodites (Ashman 1992, this study). Conversely, other studies that also controlled for cross type found no differences between the maternal sexes among the juvenile and adult fitness traits (Sakai *et al.* 1997, Emery 2001). Therefore, it appears

that maternal sex effects on offspring quality are not a component of female advantage consistent across gynodioecious taxa or even across populations within taxa. In the current study, observing offspring during later stages of development could provide additional insight into the strength and timing of maternal sex effects in *S. acaulis*, independent of seed provisioning. Undoubtedly, the large number of stages at which an effect of maternal sex might be manifested makes drawing hard conclusions difficult.

Variation among Populations

Strong differentiation among populations for germination characters, independent of inbreeding or maternal sex, suggests that some populations in this study are genetically distinct from one another. The most pronounced result involved the geographically distant population, KCT, which differed from the other populations in both the timing of germination and the final proportion of seeds that germinated (Figure 2.3). A similar pattern is also evident for floral morphology among greenhouse grown plants, with KCT individuals having a distinctly small and unique floral morphology compared to plants from the other populations (S. Keller, unpublished data). Some evidence exists indicating more inbred or self-pollinating lineages of plants also have smaller flowers compared to outcrossers (reviewed in Byers and Waller 1999). Combined with the slow rate of germination and low overall proportion germination, this suggests KCT may be more inbred than my other populations. However, the response of KCT individuals to experimental inbreeding showed no distinction from the other populations, leading me to conclude that the former traits represent divergence but not necessarily inbreeding, or that

prior inbreeding has minimal or difficult to detect effects on current levels of inbreeding depression (e.g., Latta and Ritland 1994, Shultz and Willis 1995). Additionally, the greenhouse environment may not represent realistic germination conditions in the field for each of my study populations. Differences among my populations in germination traits may also be evidence of local adaptation to environmental cues that likely differ among the geographically widespread study sites, and need not necessarily reflect differences in inbreeding history. Finally, the lack of any interaction between population and cross type supports either convergent evolution or a lack of divergence due to similar selection pressures on maternal sex and inbreeding traits that affect offspring quality among my study populations.

Summary

In conclusion, my results support the existence of inbreeding depression for seedling survival, seedling dry mass, and cumulative offspring quality among four Alaskan populations of *S. acaulis*. Maternal sex effects were confined to differences in seedling dry mass after controlling for initial seed mass, suggesting high growth rates among offspring from females compared to hermaphrodites. Offspring derived from sib crosses were not significantly reduced in quality relative to outcrosses, although a trend was observed for offspring from sib crossed hermaphrodites to have negative values of δ while those from sib crossed females generally had positive values of δ. Overall, these results indicate that inbreeding depression following selfing likely contributes to the

maintenance of females in *S. acaulis,* but that its effect is probably weak compared to the advantage females have over hermaphrodites in fruit production.

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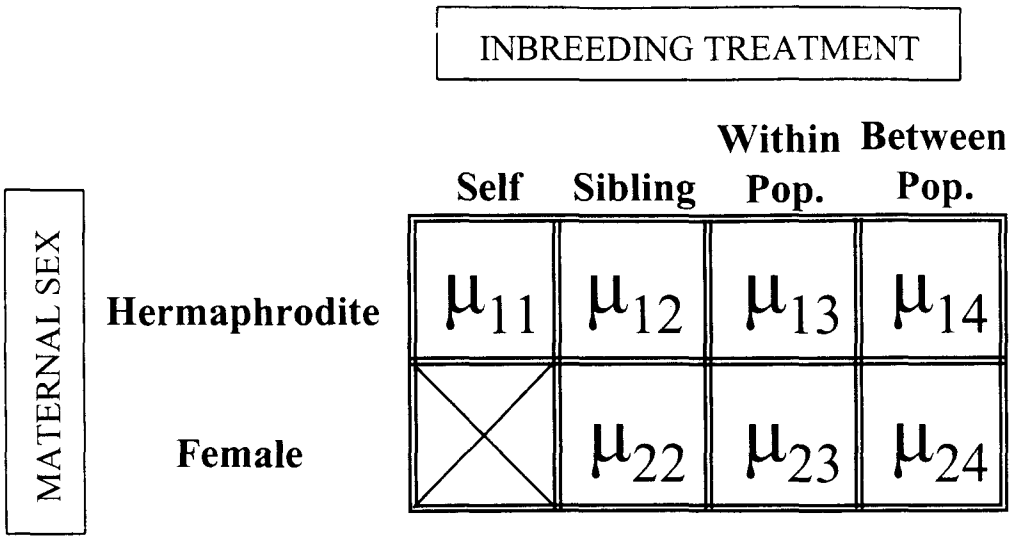

Figure 2.1. Schematic of experimental design showing the four levels of inbreeding treatment and the two maternal sex types. Parameter names are given by cell. The design was replicated for each of four populations (pop.). The structurally empty cell results from the inability of females to self.

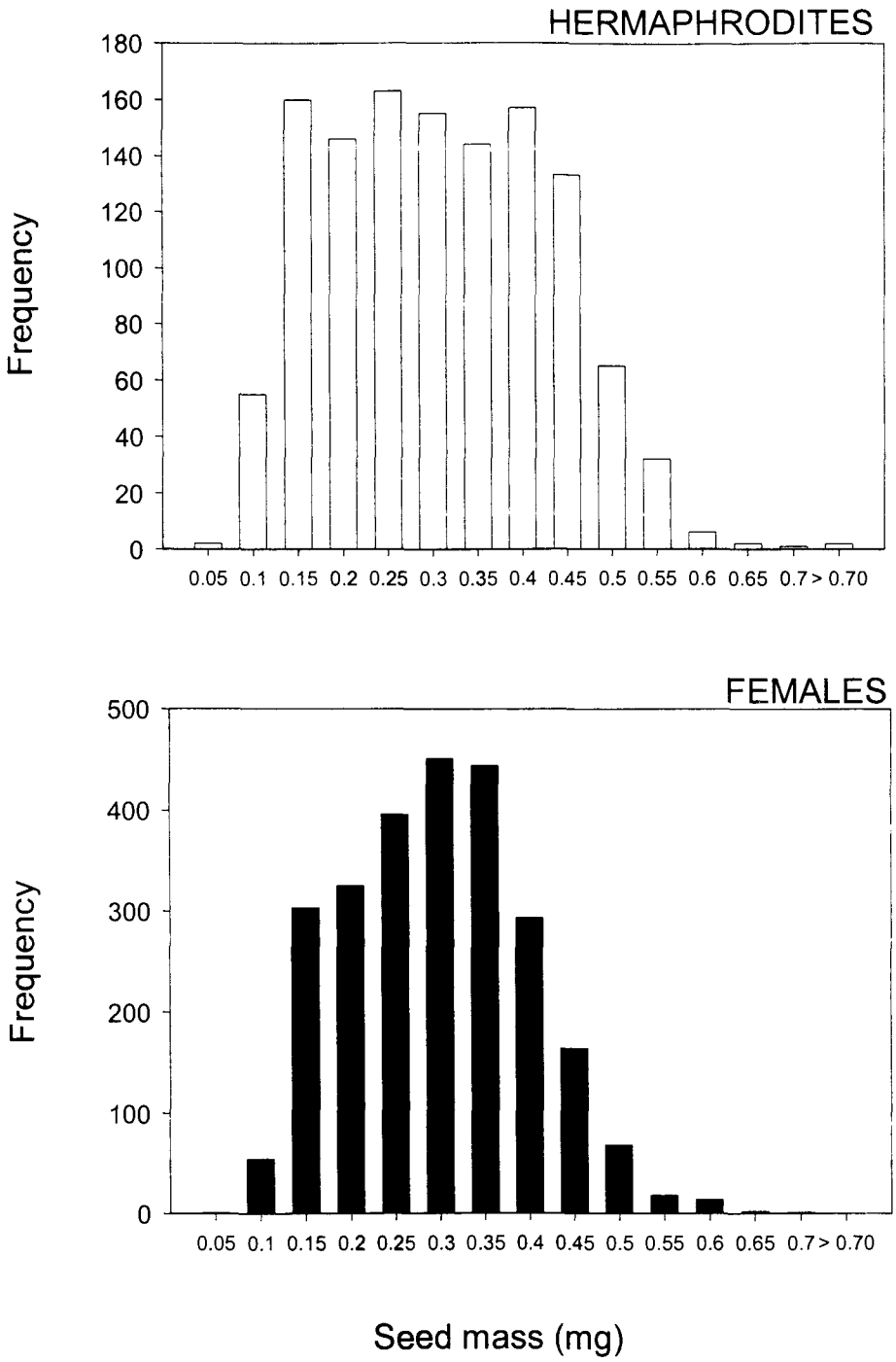

Figure 2.2. Seed mass histograms for female and hermaphrodite mothers pooled across treatments and populations.

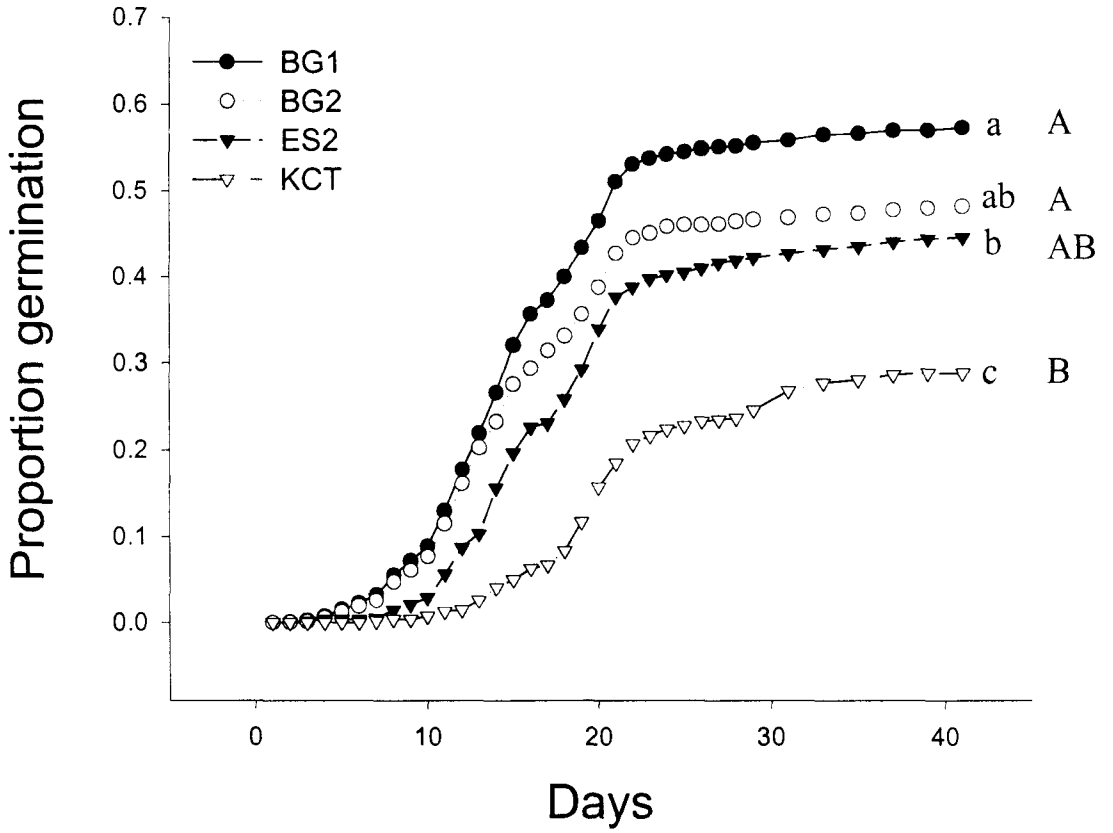

Figure 2.3. Germination curves for the study populations. Populations that share a lower-case letter do not differ in the days to germination; populations that share an upper-case letter do not differ in the final proportion of seeds that germinated ($\alpha = 0.05$). Differences among means tested using Tukey-Kramer multiple comparisons following ANOVA.

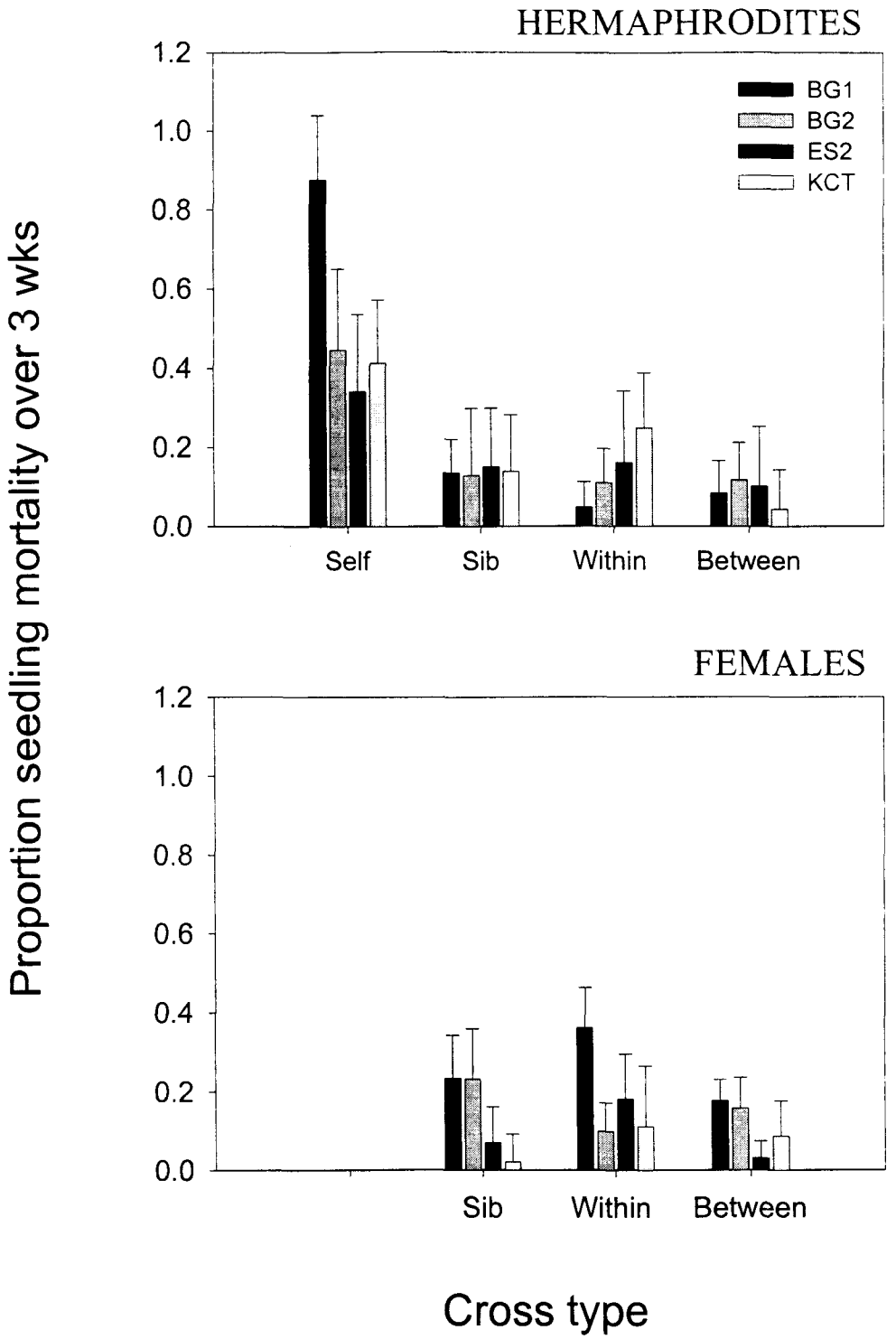

Figure 2.4. Proportion seedling mortality cumulative over the first 3 wk of growth. Values are back-transformed least squares means plus one standard error.

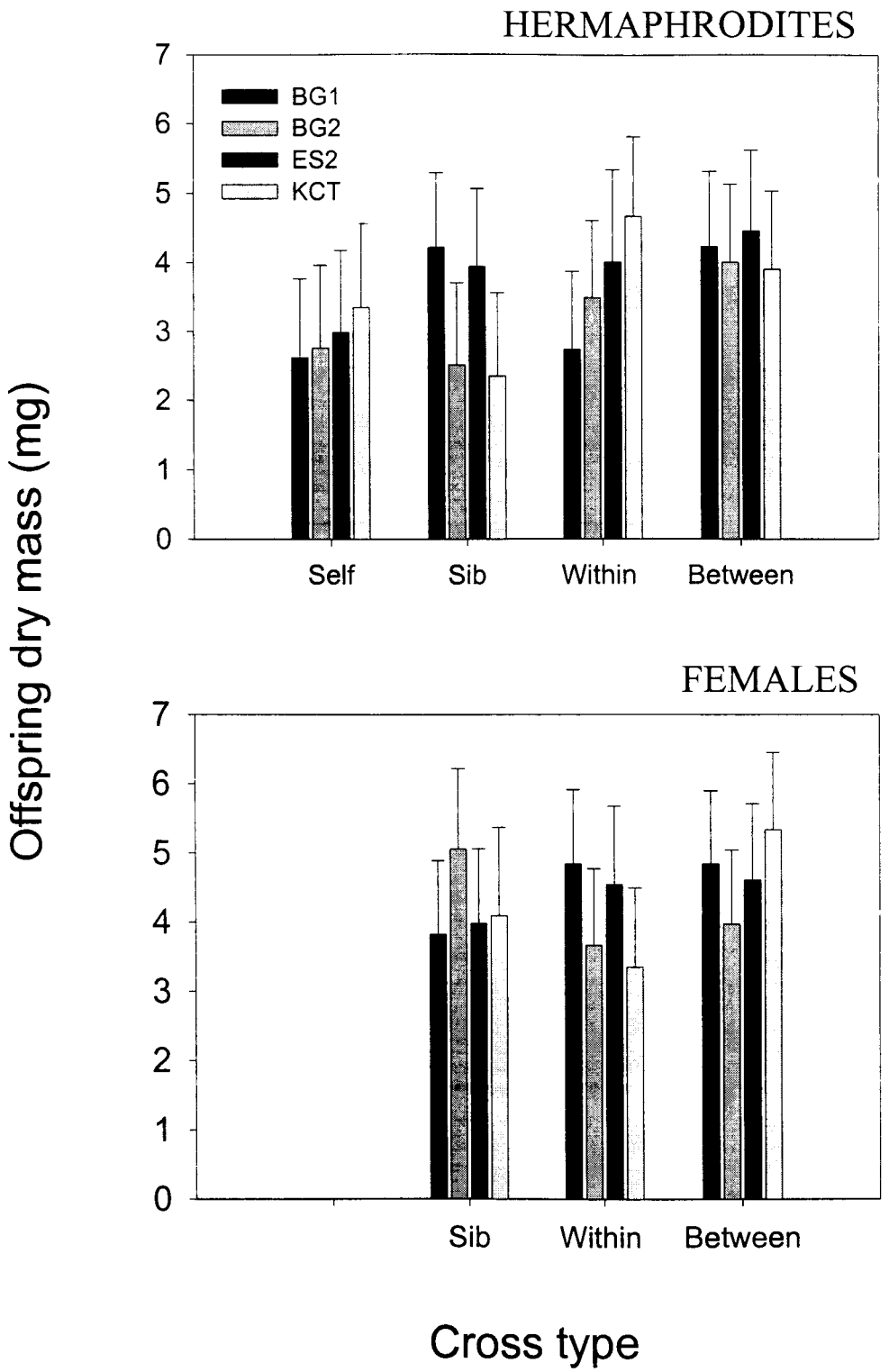

Figure 2.5. Offspring dry mass after 3 wk of growth. Values are back-transformed least squares means plus one standard error.

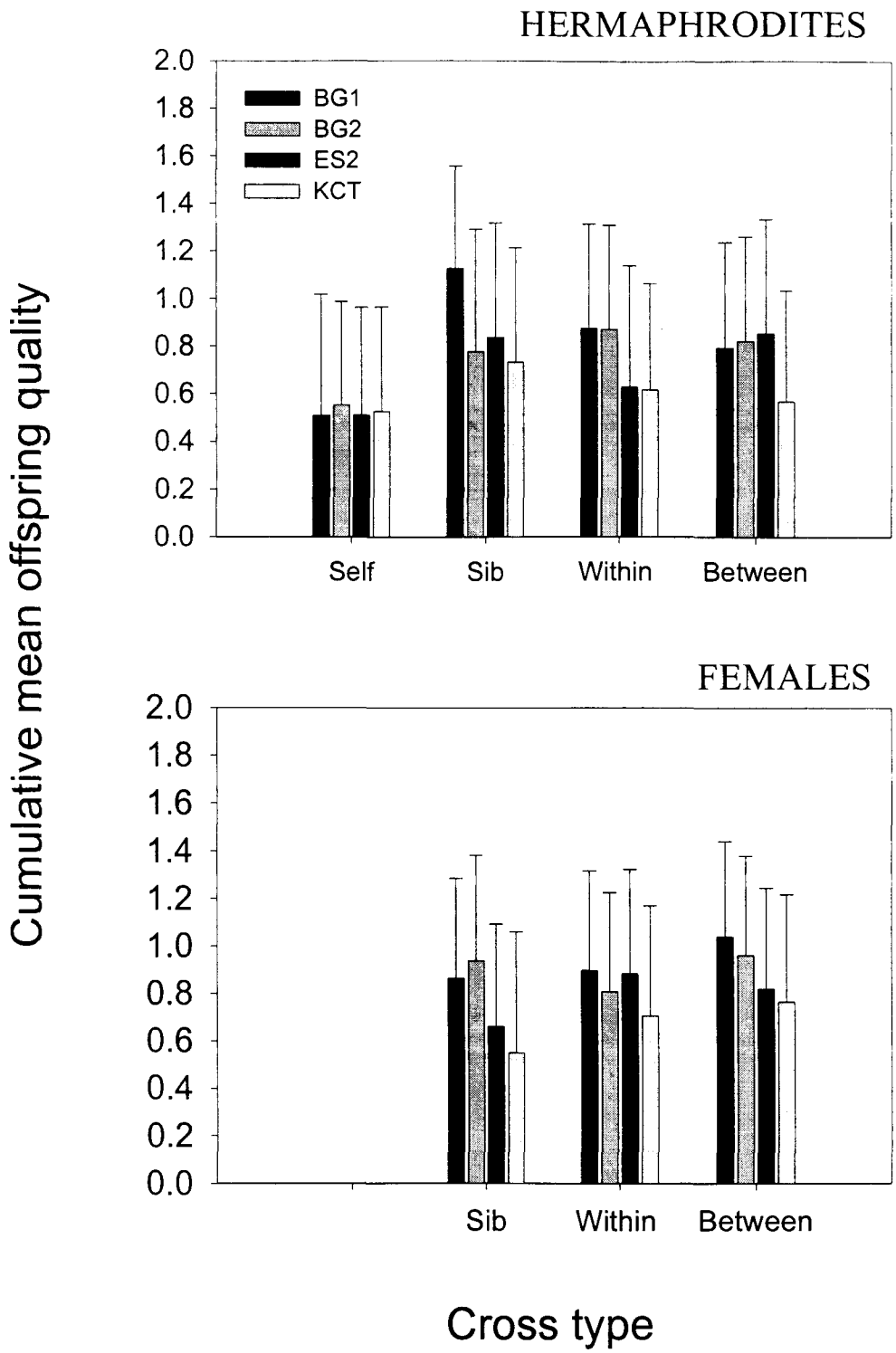

Figure 2.6. Cumulative mean offspring quality (see text for definition) after 3 wk of growth. Values are back-transformed least squares means plus one standard error.

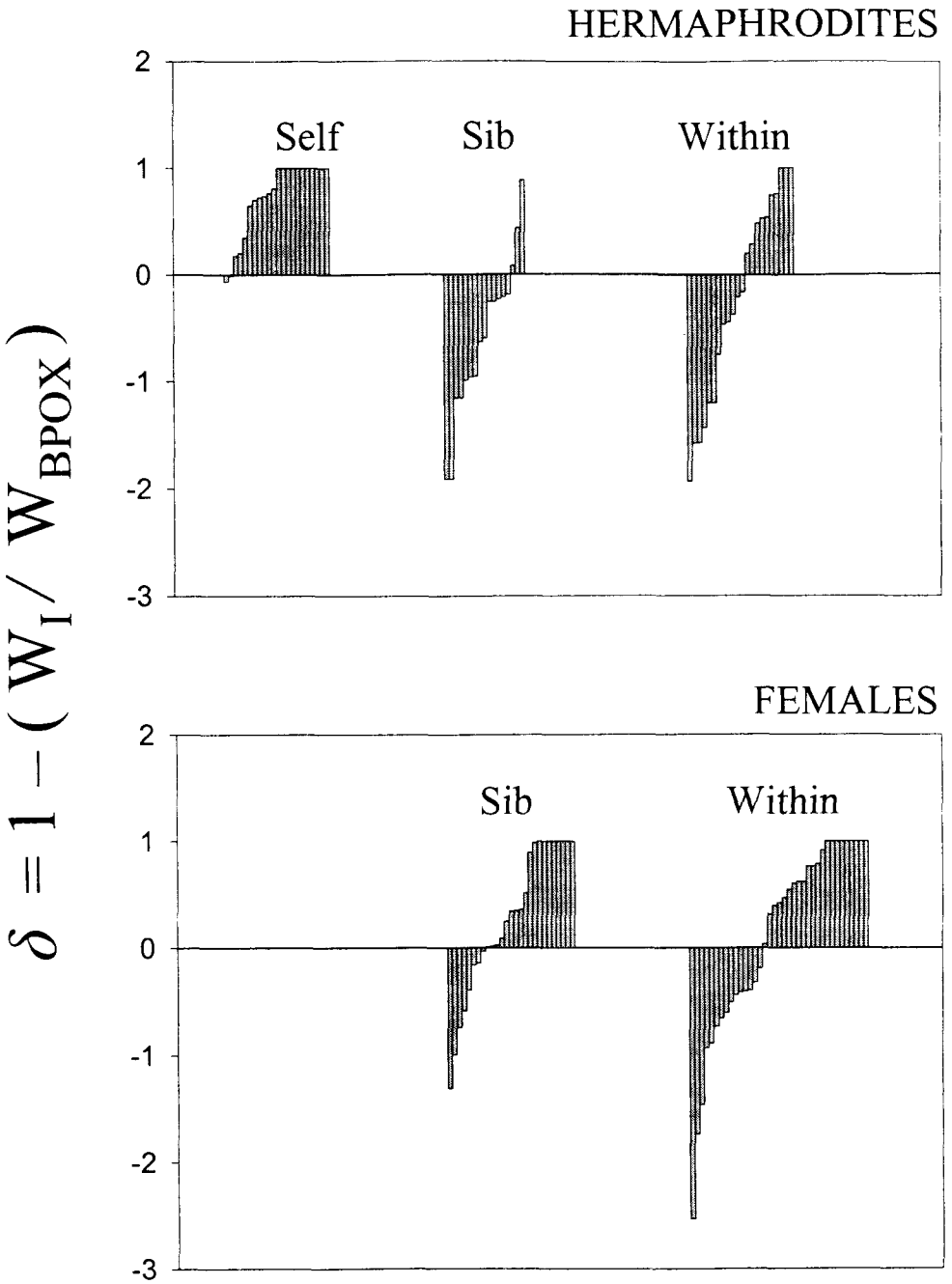

Figure 2.7. Inbreeding depression for each maternal plant standardized using the between-population outcross mean for each maternal sex and source population. Values > 0 indicate reduced fitness in inbred plants while values < 0 indicate enhanced fitness in inbred plants.

Table 2.1. Field characteristics of four Alaskan study populations of *Silene acaulis* sampled in 2001.

Population	Latitude	Longitude	Female Frequency [a]
BG1	N 63° 48′	W 148° 58′	0.69
BG2	N 63° 48′	W 148° 59′	0.52
ES2	N 65° 29′	W 145° 24′	0.55
KCT	N 61° 30′	W 142° 50′	0.57

[a] G-test of heterogeneity ($G = 11.33$, df = 3, $P = 0.01$).

Table 2.2. Hypotheses tested using pre-planned contrasts among seven combinations of sex and inbreeding treatment ("cross types"). Contrasts were tested only following a significant main effect of cross type in the ANOVA. See also Figure 2.1.

Comparison	Hypothesis
Selfs vs. Within Population Outcrosses	$2\mu_{11} - \mu_{13} - \mu_{23} = 0$
Selfs vs. Between Population Outcrosses	$2\mu_{11} - \mu_{14} - \mu_{24} = 0$
Sibs vs. Within Population Outcrosses	$\mu_{12} - \mu_{13} + \mu_{22} - \mu_{23} = 0$
Sibs vs. Between Population Outcrosses	$\mu_{12} - \mu_{14} + \mu_{22} - \mu_{24} = 0$
Within vs. Between Population Outcrosses	$\mu_{13} - \mu_{14} + \mu_{23} - \mu_{24} = 0$
Hermaphrodites vs. Females (no selfs)	$\mu_{12} + \mu_{13} + \mu_{14} - \mu_{22} - \mu_{23} - \mu_{24} = 0$
Sex * Treatment a.	$\mu_{12} - \mu_{14} - \mu_{22} + \mu_{24} = 0$
Sex * Treatment b.	$\mu_{12} - \mu_{13} - \mu_{22} + \mu_{23} = 0$
Sex * Treatment c.	$\mu_{13} - \mu_{14} - \mu_{23} + \mu_{24} = 0$

Table 2.3. ANOVA for seed mass. Model $R^2 = 0.33$.

Source	df	MS	F	*P*
Population	3	3.218	3.21	0.0244
Cross Type	6	1.280	1.28	0.2701
Population*Cross Type	18	0.777	0.78	0.7260
Maternal plant (Pop*Cross Type)	171	1.002	7.07	<0.0001
Error	3558	0.142		

Table 2.4. ANOVA for proportion mortality. Model R^2=0.22. Formulation of cross type contrasts given in Table 2.

Source	df	MS	*F*	*P*
Population	3	0.161	1.19	0.3143
Cross Type	6	0.401	2.98	0.0092
Self vs. Within Population	1	1.471	10.93	0.0012
Self vs. Between Population	1	2.24	16.62	<0.0001
Sib vs. Within Population	1	0.020	0.15	0.6995
Sib vs. Between Population	1	0.049	0.37	0.5464
Within vs. Between Population	1	0.144	1.12	0.2925
Hermaphrodites vs. Females (no selfs)	1	0.013	0.10	0.7575
Sex * Treatment a.	1	0.015	0.11	0.7387
Sex * Treatment b.	1	0.030	0.22	0.6367
Sex * Treatment c.	1	0.003	0.02	0.8779
Population*Cross Type	18	0.089	0.66	0.8415
Seed mass	1	0.681	5.06	0.0261
Error	139	0.135		

Table 2.5. ANOVA for seedling dry mass. Model R^2=0.37. Formulation of cross type contrasts given in Table 2.

Source	df	MS	*F*	*P*
Block	1	3.706	16.91	<0.0001
Population	3	0.198	0.42	0.7371
Cross Type	6	1.310	2.79	0.0137
Self vs. Within Population	1	1.355	2.89	0.0915
Self vs. Between Population	1	3.356	7.16	0.0084
Sib vs. Within Population	1	0.020	0.04	0.8379
Sib vs. Between Population	1	0.283	0.60	0.4397
Within vs. Between Population	1	0.608	1.30	0.2569
Hermaphrodites vs. Females (no selfs)	1	2.056	4.38	0.0382
Sex * Treatment a.	1	1.332	2.84	0.0943
Sex * Treatment b.	1	1.341	2.86	0.0931
Sex * Treatment c.	1	0.014	0.03	0.8640
Population*Cross Type	18	0.455	0.97	0.4981
Maternal plant (Pop*Cross Type)	130	0.469	2.14	<0.0001
Seed mass	1	54.28	247.73	<0.0001
Error	1350	0.219		

Table 2.6. ANOVA for cumulative offspring quality. Model $R^2 = 0.43$. Formulation of cross type contrasts given in Table 2.

Source	df	MS	*F*	*P*
Population	3	0.540	2.53	0.0590
Cross Type	6	0.599	2.80	0.0126
Self vs. Within Population	1	1.838	8.60	0.0038
Self vs. Between Population	1	2.429	11.37	0.0009
Sib vs. Within Population	1	0.007	0.03	0.8550
Sib vs. Between Population	1	0.017	0.08	0.7797
Within vs. Between Population	1	0.029	0.27	0.6027
Hermaphrodites vs. Females (no selfs)	1	0.051	0.24	0.6257
Sex * Treatment a.	1	0.464	2.17	0.1422
Sex * Treatment b.	1	0.288	1.35	0.2473
Sex * Treatment c.	1	0.022	0.10	0.7464
Population*Cross Type	18	0.062	0.29	0.9982
Seed mass	1	12.128	56.77	<0.0001
Error	173	0.214		

Table 2.7. Inbreeding depression for each sex and population based on cumulative offspring quality. W_I indicates the cross type used in the numerator while the between-population outcross mean for each maternal sex and population served as the denominator in calculations (see Methods). Herm = Hermaphrodite. Numbers in parentheses show the sample size of maternal plants included in each estimate.

Sex	W_I	BG1	BG2	ES2	KCT	Among population mean
Herm	Self	0.467 (2)	0.829 (8)	0.663 (5)	0.735 (7)	0.674
Herm	Sib	-1.053 (9)	-0.613 (2)	0.087 (3)	0.145 (3)	-0.358
Herm	Within	-0.476 (7)	-0.013 (7)	0.472 (2)	-0.384 (6)	-0.100
Female	Sib	0.284 (11)	0.125 (6)	0.294 (8)	0.617 (2)	0.330
Female	Within	-0.040 (13)	0.129 (13)	0.175 (7)	0.415 (4)	0.170

CONCLUSIONS

In Chapter 1, I investigated the variation in the frequency of females across space and time, as well as fruit production and seed viability. I found that Alaskan populations of *Silene acaulis* show considerable variability in the spatial distribution of females and hermaphrodites, with most of the variation distributed between populations. Spatial patchiness of females within some populations indicates that the genes controlling sex expression may be finely structured, but this effect is modest overall in scale and frequency of occurrence. Heterogeneity among populations in sex ratio may represent differences in the diversity and equilibrium frequency of sex determining genes, as the majority of populations show no sign of nonequilibrium sex ratios as inferred from size class distributions. When the frequency of the sexes does differ across size classes, the trend is for fewer females among the smaller plants, indicating a decrease in female frequency over time that may be due to an increasing frequency of nuclear restorers following invasion by a CMS gene. Females far exceed hermaphrodites in fruit production, with the magnitude of the advantage more than compensating for their loss of male fitness. Additionally, the presence of females may actually benefit the population by enhancing the outcrossing rate and increasing progeny viability. Overall, the genetics of male sterility in *S. acaulis* appear to create spatially complex and variable patterns of sex expression that may have important implications for seed fitness in natural populations.

In Chapter 2, I explored the effects of inbreeding and maternal sex as potential determinants of female advantage in offspring quality. My results support the existence

of inbreeding depression for seedling survival, seedling dry mass, and cumulative offspring quality among four Alaskan populations of *S. acaulis*. Maternal sex effects were confined to differences in seedling dry mass after controlling for initial seed mass, suggesting high growth rates among offspring from females compared to hermaphrodites. Offspring derived from sib crosses were not significantly reduced in quality relative to outcrosses, although a trend was observed for offspring from sib crossed hermaphrodites to have negative values of δ while those from sib crossed females generally had positive values of δ. These results indicate that inbreeding depression following selfing likely contributes to the maintenance of females in *S. acaulis,* but that its effect is probably weak compared to the fecundity advantage females have over hermaphrodites in fruit production.

Overall, the *Silene acaulis* populations I studied show evidence of being cryptically dioecious. The low fruit production and seed quality of hermaphrodites suggest they may be functioning primarily as pollen donors. Alaskan populations of *Silene acaulis* may thus represent an example of a gynodioecious species that is approaching the evolution of complete separation of the sexes.